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SOME EFFECTS OF *IN VIVO* ADMINISTERED ESTROGENS ON GLUCOSE METABOLISM AND ADRENAL CORTICAL SECRETION *IN VITRO*

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ABSTRACT

Normal young male Wistar rats were given daily subcutaneous depot doses of estrogens for periods from 1 to 15 days. After starving for about 20 hours, the animals were decapitated and samples of liver taken for total lipid and glycogen determinations. The diaphragms were excised, incubated *in vitro* with glucose alone or with glucose plus insulin. Glucose uptake and glycogen deposition were measured. The adrenals were also incubated *in vitro* and the glucocorticoid output measured.

The estrogenic compounds given *in vivo* were found to increase the *in vitro* response of diaphragm muscle. For example, the administration of ethinyl estradiol for 12 days increased the glucose utilization and glycogen formation in diaphragm by as much as 60% compared to controls.

The insulin effect on glucose uptake (increase of stimulated over unstimulated) in diaphragms from ethinyl estradiol treated animals was often increased 2–3 times. The insulin effect on glycogen deposition was also increased but to a lesser extent. Estradiol and stilbestrol were less effective than ethinyl estradiol in increasing the insulin effect.

Liver glycogen levels were higher and liver total lipid levels slightly lower in the estrogen treated rats.

The secretion of corticoids *in vitro* was significantly lower in adrenals from estrogen treated rats. The lowest output was found in adrenals from the ethinyl estradiol treated animals. The concentration of corticoids remaining after incubation was also found to be lower in the adrenal tissues of estrogen treated rats.

A mechanism of action is postulated for the insulin potentiating effect of estrogens.

IT IS well substantiated that the pituitary and adrenals in some way modify the effect of insulin on metabolism. Removal of either the pituitary or adrenals increases insulin sensitivity. In addition it is appre-

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ciated that depancreatized animals are in much better metabolic balance when the pituitary and/or adrenals are also removed. A complete review of the literature of these interrelationships is beyond the scope of this paper. The interested reader is referred to several thorough review articles (1, 2, 3, 4).

Kinsell (5) found that hypophysectomy changed a unstable human diabetic requiring 70 U. insulin/24 hours to a mild diabetic requiring 10–15 U./24 hours. Glucocorticoids and somatotrophin have been shown to possess antiinsulin activity (6). It has been postulated by Randle and Young (7) that growth hormone or some associated factor in crude pituitary preparations may be the diabetogenic factor that inhibits insulin action. Stadie *et al.* (8) showed that isotopically labeled insulin bound to the diaphragm was much more active in accelerating glycogen synthesis in the hypophysectomized rat. Krahl and Park (9) also demonstrated that growth hormone, when injected into hypophysectomized rats 3 hours prior to diaphragm removal, produced a 40–50% depression of subsequent glucose uptake by such diaphragms *in vitro*.

Whether growth hormone can exert its inhibitory effect directly on muscle is not clear. Randle (10) found a direct effect on diaphragm muscle *in vitro* only in the Stadie-Zapp sodium buffer and not in the Gey and Gey balanced salt solution. On the other hand, Bron, and Fisher (11), utilizing the Gey and Gey buffer system, have recently shown that Wilhelmi growth hormone depresses the glucose uptake of the perfused rat heart, both in the absence and in the presence of 2 milliunits of insulin. Bornstein and Park (12) reported that inhibition of insulin action may be mediated by means of a lipoprotein carried in the serum and that the level of this lipoprotein increases in the diabetic. Lately, however, Bornstein (13) showed this effect to be nonspecific since several enzymes other than those utilizing glucose are inhibited by serum lipoprotein fractions. Baird and Bornstein (14) have recently demonstrated that insulin is present in the plasma of patients in diabetic coma but is masked by an antagonist which can be separated.

We have been interested in assessing the effect of sex hormones on metabolism because of the sex difference in susceptibility to diseases such as cardiovascular disorders. Ingle (15) reported that administration of estrogens, if anything, increased the severity of diabetes. On the other hand, Lewis *et al.* (16) demonstrated that estrogens, when given over a six-months period, lessen the severity of the diabetic state in subtotally depancreatized rats presumably by stimulating the regeneration of pancreas tissue. Noble (17, 18) demonstrated that treatment of rats with stilbestrol produces atrophy of the gonads and accessory sex organs and inhibition of growth, presumably by inhibition of pituitary function. It has been shown by McKerns (19) that certain synthetic estrogens inhibit adrenal corticoid

secretion *in vitro* as well as gonad secretion when injected *in vivo*. In the *in vivo* experiments, the resultant pituitary enlargement was thought by histological examination to have increased corticotrophin and gonadotrophin but decreased growth hormone secretion. Griffiths, Marks and Young (20) demonstrated that treatment with stilbestrol leads to an increase of liver glycogen in the fasting rat. This increase was apparently not due to a transfer of glycogen from the muscles to the liver. Griffiths and Young (21) also showed that the administration of estrogens increased the amount of extractable insulin obtained from the pancreas.

Whatever the mechanism of action of insulin and however antagonism of the pituitary is involved it seemed that an assessment of glucose metabolism in muscle after *in vivo* pretreatment might reveal compounds effective in potentiating insulin action. This paper is concerned with a preliminary study of the effect of estrogen administration *in vivo* on glucose metabolism. The effect of estrogen administration on adrenal cortical output *in vitro* is also presented herein.

MATERIALS AND METHODS

Animal Dosing. Estrogens were given daily as subcutaneous suspension in 0.2 ml. of 0.5% carboxymethylcellulose, 0.4% Tween 80 in 0.9%NaCl. In certain experiments stilbestrol was administered in cottonseed oil. Normal male rats obtained from Royalhart Lab. Animals, New Hampton, New York, were used in all experiments.

Rat Diaphragm Method. Randle (22, 23) has improved the rat diaphragm technique for the assessment of insulin action *in vitro*. Further improvements have been made in this laboratory so that a statistically sound 4 point micro assay for insulin can be run (24). Furthermore, physiological amounts of insulin are used at about the midpoint of the dose-response curve. Many workers have used such high levels of insulin that the possibility of masking any inhibiting or potentiating effects must have been introduced. Each experiment is made up of 16 male rats, consisting of 4 littermates from 4 different litters of the same age. Three treatments are compared with a control group. The animals are distributed such that a littermate is on each treatment. The animals are allowed to feed *ad libitum* on a stock diet of Wayne lab. blocks obtained from Allied Mills, Inc., Chicago, Illinois, 4 littermates to a cage, and are starved for approximately 20 hours following the last day of treatment. In experiments in which just stilbestrol treatment was compared to controls, littermates were distributed equally between both groups.

At the end of the treatment period the rats are decapitated without anesthesia and the diaphragms removed quickly and carefully. Four animals are killed in rapid succession and the diaphragms worked up and placed in the Dubnoff incubator before proceeding to the next group of four. After excision the diaphragms are rinsed and left to soak for 10 minutes (from the time the fourth diaphragm is removed) in 25 ml. of ice cold Gey and Gey buffer (25) without glucose. The diaphragms are then gently blotted on filter paper. The central muscle and membrane is cut out leaving two halves. The hemidiaphragms are weighed and one hemidiaphragm is placed in a 10 ml. beaker containing 3 ml. of ice cold Gey and Gey buffer with 200 mg.% glucose. The other hemidiaphragm is placed in a beaker containing buffer with glucose and 1 milliunit insulin per ml. The beakers are then left to stand on ice for a further 5 minutes. This procedure reduces the variability and greatly increases the insulin response of the tissue. The beakers containing the hemidiaphragms are then placed in a Dubnoff metabolic shaker (130 cycles/min.)

at 37° C for 60 minutes in a gas phase of 95% O_2 and 5% CO_1. After incubation the beakers are removed and placed in a tray of cracked ice. The hemidiaphragms are drained and rinsed into the beakers with 1 ml. water and immediately dropped into 2 ml. 30% KOH in a 15 ml. centrifuge tube. The tube is then fitted with a condenser and heated in a boiling water bath for 30 minutes. Glycogen is measured by the procedure described by Stadie (26).

Glucose is assayed by the Nelson method (27). Glucose uptake is calculated as the difference between the glucose level in incubation medium not containing diaphragm and glucose level of incubation medium containing diaphragm. Glucose uptake and glycogen deposition are expressed as mg. glucose or mg. glycogen per g. diaphragm. The statistical significance of any changes is calculated by analysis of variance with a P value of 0.05 or less taken as indicative of a significant difference.

Measurement of Adrenal Corticoid Secretion. The method of McKerns and Nordstrand was used (28). Essentially the method consists of incubating quartered adrenal pieces for 2 hours in a buffered bicarbonate medium in a gas phase of 95% O_2 and 5% CO_2. After incubation, the buffer medium is removed from the tissues and steroids secreted into the buffer medium are extracted with methylene chloride. The adrenal tissue is homogenized and likewise extracted. The corticoid levels in the methylene chloride extracts are measured by the corrected U.V. absorption at 240 mμ (29) or by the blue tetrazolium reaction as described by Henley (30).

When the steroids were chromatographed, four or more methylene chloride extracts of a given treatment were pooled and the solvent blown off with N_2. The residue was dissolved in methanol and extracted with an equal volume of heptane to remove lipids. The methanol layer was taken off and blown down with N_2. The residue was redissolved in methylene chloride and applied to the paper strip (31). Chromatography was carried out on Whatman No. 1 paper in the toluene-propylene glycol system described by Burton *et al.* (32). The paper was previously wetted with propylene glycol:methanol, 1:1. The paper strips were read in the U.V. on a Photovolt paper scanner and the absorption curves plotted. Indidividual spots were sometimes eluted from the paper with methanol and quantitatively assayed by the above mentioned methods. By using these techniques, the findings obtained by the direct ultraviolet determination on the methylene chloride extracts were confirmed.

The statistical significance of any changes was estimated by an analysis of variance of the data with a P value of 0.05 or less taken to indicate a significance difference.

Liver Glycogen and Total Lipid Determinations. Liver glycogen was determined on 60–100 mg. samples by the method mentioned above for muscle glycogen. Liver total lipid was measured on approximately 1.0 g. samples by the method of Shipley *et al.* (33).

RESULTS

Effect of Stilbestrol, Ethinyl Estradiol or Estradiol Administered Daily for 12–15 Days:

Effect on Body Weight. The average starting and final weights for a 12–15 day estrogen injection period are given in Table 1. A slight depression in body weight was observed in the estrogen treated animals.

Effect of Estrogen Administration on Glucose Uptake and Glycogen Deposition in Diaphragm in Vitro. An example of the effect of estrogen treatment on glucose uptake *in vitro* is given in Table 2. It can be seen that the largest glucose uptake was obtained in the diaphragms of stilbestrol and ethinyl estradiol treated rats. These two treatment groups also had the greatest

TABLE 1. EFFECT OF ESTROGEN ADMINISTRATION ON BODY WEIGHT

Each value represents the average weight in grams of 4 rats. Final weights were measured after a 20-hour fasting period.

Exper. No.	Control		Stilbestrol		Ethinyl estradiol		Estradiol	
	Start	Final	Start	Final	Start	Final	Start	Final
112	107	117	108	114.8	95	91.5	105	104.8
118	114	133	117	129.3	129.3	102	93	110.3
147	111.8	149	117	107	105.3	99	120	119
153	137	202	124	123	120	117	124	125

insulin effect (the increase of insulin stimulated glucose uptake over unstimulated). The corresponding glycogen formation in these diaphragms is shown in Table 3.

TABLE 2. (EXP. NO. 147). EFFECT OF *in Vivo* PRETREATMENT WITH ESTROGENS (200 γ/DAY FOR 15 DAYS) ON THE SUBSEQUENT GLUCOSE UPTAKE IN HEMIDIAPHRAGM *in Vitro*.

Glucose uptake as mg./g. diaphragm. Insulin at 1 milliunit/ml. Incubation time 1 hour.

	Treatment groups							
	1 Control		2 Stilbestrol		3 Ethinyl estradiol		4 Estradiol	
	Unstim.	Insulin stim.	Unstim.	Insulin stim.	Unstim.	Insulin stim.	Unstim.	Insulin stim.
	5.36	5.19	6.31	9.56	6.0	9.40	4.37	5.90
	4.83	4.71	5.21	6.66	7.68	7.53	3.78	5.10
	4.68	5.70	5.36	7.68	5.87	8.51	5.12	7.34
	4.11	5.52	5.97	6.39	6.52	8.48	5.23	6.21
means	4.75	5.28	5.71	7.57	6.52	8.48	4.63	6.14
Insulin effect	0.53		1.86		1.96		1.51	

Analysis of variance

Nature variation	Degrees freedom	Sum squares	Mean square	F-Ratio
Total	31	67.44		
Treatment groups	3	31.56	10.52	16.8†
Insulin stim.	1	17.23	17.23	27.5†
Treatments × Insulin stim.	3	2.54	0.85	1.4
Litters	3	2.96	0.99	1.6
Error S^2	21	13.15	0.626	

S.E. mean = ±0.398

t between unstim. and insulin stim. of control = 0.95
t between unstim. and insulin stim. of stilbestrol = 3.32†
t between unstim. and insulin stim. of ethinyl estradiol = 3.39†
t between unstim. and insulin stim. of estradiol = 2.88†

t between insulin stim. of 1 and 2 = 4.09
t between insulin stim. of 1 and 3 = 5.71‡
t between insulin stim. of 1 and 4 = 2.15*

* = $P < 0.05$.
† $P < 0.01$.
‡ $P < 0.001$.

TABLE 3. (EXP. NO. 147). EFFECT OF *in Vivo* PRETREATMENT WITH ESTROGENS (200 γ/DAY FOR 15 DAYS) ON THE SUBSEQUENT GLYCOGEN FORMATION IN THE DIAPHRAGM *in Vitro*

Results as mg. glycogen/g. diaphragm. Insulin at 1 milliunit/ml. Incubation time 1hour.

Treatment groups								
1 Control		2 Stilbestrol		3 Ethinyl estradiol		4 Estradiol	
Unstim.	Insulin stim.	Unstim.	Insulin stim.	Unstim.	Insulin stim.	Unstim.	Insulin stim.
2.10	2.61	2.67	3.74	2.43	3.63	1.75	2.04
1.88	2.70	3.00	3.53	2.45	3.04	1.63	2.11
1.93	2.39	2.55	3.23	2.24	3.02	2.19	2.87
1.77	2.69	3.00	3.47	2.37	3.23	1.77	2.49
means 1.92	2.60	2.81	3.49	2.37	3.23	1.84	2.38
Insulin effect 0.68		0.68		0.86		0.54	

Analysis of variance

Nature variation	Degrees freedom	Sum squares	Mean square	F-Ratio
Total	31	10.81		
Treatment groups	3	5.60	1.87	31.1†
Insulin stim.	1	3.82	3.82	63.7†
Treatments × insulin stim.	3	0.10	0.03	0.56
Litters	3	0.03	0.01	0.17
Error S^2	21	1.26	0.06	

S.E. mean = ±0.12

t between unstim. and insulin stim. of control	= 4.00‡
t between unstim. and insulin stim. of stilbestrol	= 4.00‡
t between unstim. and insulin stim. of ethinyl estradiol	= 5.06‡
t between unstim. and insulin stim. of estradiol	= 3.18†
t between insulin stim. of 1 and 2	= 5.24‡
t between insulin stim. of 1 and 3	= 5.24‡
t between insulin stim. of 1 and 4	= N.S.

† = $P < 0.01$.
‡ = $P < 0.001$.

Further results on glucose uptake are given in Table 4. The increase in glucose uptake is especially marked in insulin stimulated diaphragms from ethinyl estradiol treated rats. Table 5 shows similar responses to the estrogen treatments in the glycogen formation by the diaphragms.

Effects on Liver Glycogen and Lipid Levels. Liver glycogen levels determined immediately after decapitation are given in Table 6. The glycogen values are considerably higher than the controls in the livers from the estrogen treated animals except in the case of stilbestrol treatment in experiment 112.

Table 7 shows that liver lipid levels were slightly and usually significantly lower in the livers from estrogen treated animals. Glucose levels in blood were also determined in certain experiments but no differences between control and estrogen treated animals were observed.

Effect of Estrogen Treatment on Adrenal Cortical Secretion. The effect of estrogen treatments on corticoid secretion of the adrenals *in vitro* is presented in Table 8. It can be seen that all the *in vivo* estrogen treatments significantly lowered the total *in vitro* adrenal secretion as measured by the ultraviolet absorption method. Ethinyl estradiol treatment had the greatest inhibitory effect.

Corticoid levels were also determined in pooled tissue extracts of the adrenals from each treatment at the end of the incubation period. As shown in Table 8, the levels of corticoids were lower in the adrenals of the estrogen treated rats suggesting an impairment in synthesis from endogenous precursors in these groups. An interesting alternative viz. that corticoid catabolism in adrenals of estrogen treated rats is enhanced, should also be investigated.

Figure 1 shows an example of the amounts and relative positions of steriods extracted from media fluid after adrenal incubation. Chromatography was carried out in the toluene-propylene glycol system (32). The amounts of steroids on the strips were estimated in a paper strip scanner

TABLE 4. EFFECT OF *in Vivo* PRETREATMENT WITH ESTROGENS ON THE SUBSEQUENT GLUCOSE UPTAKE IN HEMIDIAPHRAGM *in Vitro*

Glucose uptake as mg./g. diaphragm. Insulin at 1 milliunit/ml. Incubation time 1 hour.

Experiment No. and dose regime	1 Control		2 Stilbestrol		3 Ethinyl estradiol		4 Estradiol	
	u.	s.	u.	s.	u.	s.	u.	s.
118 (200 γ daily for 12 days)	5.23	5.86	5.20	7.13	6.23	8.11	6.49	6.86
Insulin effect:	0.63		1.93		1.88		0.37	
	S.E. mean = ±0.39							
153 (200 γ daily for 15 days)	2.67	3.63	4.28	5.22	4.58	6.66	4.38	5.38
Insulin effect	0.96		0.94		2.08		1.00	
	S.E. Mean = ±0.44							

	Experiment No.	
	118	153
t between u. and s. of control	= 1.14	1.55
t between u. and s. of stilbestrol	= 3.51†	1.52
t between u. and s. of ethinyl estradiol	= 3.42†	3.35†
t between u. and s. of estradiol	= 0.67	1.61
t between s. of 1 and 2	= 2.31*	2.56*
t between s. of 1 and 3	= 4.08‡	4.89‡
t between s. of 1 and 4	= 1.82*	2.82*

Note: Each value is the mean glucose uptake of 4 hemidiaphragms assayed individually.
u. = unstimulated.
s. = stimulated by insulin.

* = $P < 0.05$.
† = $P < 0.01$.
‡ = $P < 0.001$.

TABLE 5. EFFECT OF *in Vivo* PRETREATMENT WITH ESTROGENS ON THE *in Vitro* GLYCOGEN FORMATION OF RAT HEMIDIAPHRAGM

Results as mg. glycogen/g. diaphragm. Insulin at 1 milliunit/ml. Incubation time 1 hour.

Experiment No. and dose regime	1 Control		2 Stilbestrol		3 Ethinyl estradiol		4 Estradiol	
	u.	s.	u.	s.	u.	s.	u.	s.
118 (200γ daily for 12 days)	1.81	2.10	2.24	2.90	2.38	3.33	2.38	3.18
Insulin effect:	0.29		0.66		0.95		0.80	
153 (200 γ daily for 15 days)	1.79	2.54	2.13	2.87	2.01	2.73	1.83	2.76
Insulin effect:	0.75		0.74		0.72		0.93	

S.E. mean = ±0.16

	Experiment No. 118	Experiment No. 153
t between u. and s. of control =	0.88	2.42
t between u. and s. of stilbestrol =	2.00*	2.38*
t between u. and s. of ethinyl estradiol =	2.88†	2.32*
t between u. and s. of estradiol =	2.43*	3.00†
t between s. of 1 and 2 =	2.43*	1.43
t between s. of 1 and 3 =	3.74†	0.83
t between s. of 1 and 4 =	3.28†	0.96

Note: each value is the mean glycogen formation of 4 hemidiaphragms assayed individually.

u. = unstimulated.
s. = insulin stimulated.

* = P < 0.05.
† P < 0.01.

TABLE 6. EFFECT ON LIVER GLYCOGEN LEVELS OF RATS GIVEN ESTROGENS SUBCUTANEOUSLY (220 γ/DAY 12–15 DAYS)

Animals fasted 20 hours before killing. Results expressed as mg./gm. liver and represent the mean of 4 determinations.

Experiment No.	Treatment groups 1 Control	2 Stilbestrol	3 Ethinyl estradiol	4 Estradiol
112	0.85	0.46	4.66	2.74
	S.E. = ±0.6			
	t between 1 and 2 = 0.46 t between 1 and 3 = 4.48† t between 1 and 4 = 2.22*			
118	2.56	6.14	3.35	3.56
	S.E. = ±1.5			
	Differences from control not significant			
153	0.46	9.11	7.67	6.01
	S.E. = ±1.76			
	t between 1 and 2 = 3.46† t between 1 and 3 = 2.88* t between 1 and 4 = 2.22*			

* P < 0.05.
† = P < 0.01.

TABLE 7. EFFECT ON LIVER TOTAL LIPID LEVELS OF ESTROGEN TREATED RATS (200 γ/DAY FOR 12–15 DAYS)

Results are expressed as mg./gm. liver and represent the mean of 4 determinations.

Experiment No.	Treatment groups			
	1 Control	2 Stilbestrol	3 Ethinyl estradiol	4 Estradiol
118	66.4	76.9	64.9	62.4
		S.E. = ±1.7		
		differences not significant		
122	67.8	64.1	62.9	61.2
		S.E. = ±1.95		
		t between 1 and 2 = 1.34		
		t between 1 and 3 = 1.77		
		t between 1 and 4 = 2.38*		
147	68.6	62.6	67.0	61.1
		S.E. = ±1.35		
		t between 1 and 2 = 3.13*		
		t between 1 and 3 = 0.83		
		t between 1 and 4 = 3.91†		
153	78.0	71.7	70.8	70.1
		S.E. = ±2.00		
		t between 1 and 2 = 2.22*		
		t between 1 and 3 = 2.54*		
		t between 1 and 4 = 2.78*		

* = P < 0.05.
† = P < 0.01.

TABLE 8. EFFECT OF *in Vivo* PRETREATMENT WITH ESTROGENS (200 γ/DAY FOR 15 DAYS) ON *in Vitro* ADRENAL CORTICOID SECRETION AND ON THE CORTICOID LEVELS REMAINING IN THE POOLED ADRENAL TISSUE AFTER INCUBATION

Results expressed as γ cortisone/100 mg. adrenal tissue. Corticoids determined by ultraviolet absorption.

Experiment No.	Corticoid determined in	1 Control	2 Stilbestrol	3 Ethinyl estradiol	4 Estradiol
147	Incubation media	24.0	15.1	10.8	18.8
		S.E. mean = ±1.62			
		t between 1 and 2 = 3.86†			
		t between 1 and 3 = 5.74†			
		t between 1 and 4 = 2.26*			
	Pooled adrenal tissue	27.9	25.7	24.1	25.6
153	Incubation media	29.2	15.2	11.0	18.4
		S.E. mean = ±0.5			
		t between 1 and 2 = 19.36‡			
		t between 1 and 3 = 25.72‡			
		t between 1 and 4 = 15.35‡			
	Pooled adrenal tissue	43.0	29.5	27.6	39.5

Note: Each value is the mean of 4 determinations.

* = P < 0.05.
† = P < 0.01.
‡ = P < 0.001.

by U.V. absorption. Spots were then visualized by the blue tetrazolium reaction.

In agreement with Heard *et al.* (34) four distinct U.V. fluorescing areas were found on chromatograms of extracts of adrenal incubation media from control rats. The first and fourth areas corresponded to the positions of 17-hydroxycorticosterone (comp. F) and corticosterone (comp. B) respectively and gave an ultraviolet fluorescence as well as a blue tetrazolium color. The second spot, which was not identified, also gave an ultraviolet fluorescence and a blue tetrazolium color. The third spot, also unidentified, fluoresced under ultraviolet light but gave no tetrazolium color. Most of

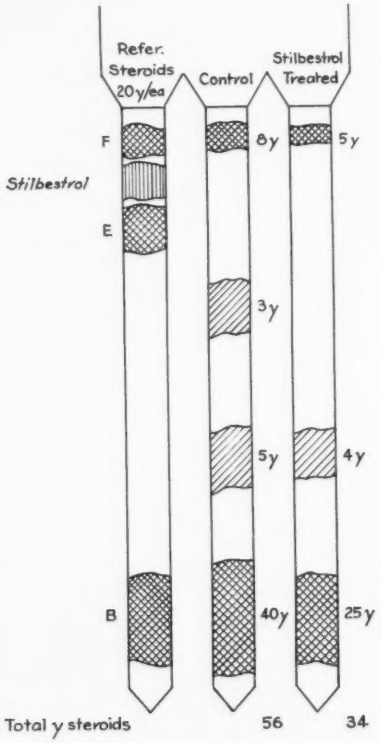

FIG. 1. Effect of *in vivo* stilbestrol treatment on the adrenal steroid secretion *in vitro*. Adrenal from 8 stilbestrol treated (total adrenal weight 243.3. mg.) and 8 control rats (total adrenal weight 233.8 mg.) were removed after 14 days treatment. The adrenals were incubated *in vitro* and the extracts of the incubation media chromatographed in a toluene-propylene glycol system. The amount of steroids was estimated with a paper strip scanner in the ultraviolet. Spots were visualized by the blue tetrazolium reaction. The Third spots (5γ on the control and 4γ on the stilbestrol strip)fluoresced but gave no tetrazolium reaction.

the U.V. fluorescence and blue tetrazolium color was found in the area of corticosterone.

Treatment with estrogens reduced all four areas on chromatograms. The adrenals were obtained from eight stilbestrol treated rats which received 100 γ/day for 15 days.

Stilbestrol gave a bright green fluorescence in the reference strip under U.V. light in the area between comp. F and comp. E. At very high dose levels (250 γ/100 gm.) stilbestrol could be identified on chromatograms of extracts from adrenal tissue and incubation media. A more extensive presentation of studies on quantitative estimations of adrenal steroids after estrogen and other steroid treatments will be the subject of a future paper.

The Effect of One Dose of Stilbestrol:

Rats of approximately 180 g. were given 300γ stilbestrol/100 gm. subcutaneously in 0.2 ml. cottonseed oil. Food was withdrawn and 18 hours later the animals were killed. The subsequent glucose uptake by the diaphragm *in vitro* was slightly but not significantly enhanced in both stimulated and insulin stimulated diaphragms. The glycogen deposition was similarly but not significantly increased in both unstimulated and insulin stimulated diaphragms from stilbestrol treated animals. The stilbestrol dose significantly increased the liver glycogen level. The results are given in Table 9.

The Effect of Administering a 500γ Dose Stilbestrol/100 gm. for 14 Days:

The average body weights of eight stilbestrol treated animals were 71 gm. and 90 gm. for starting and final weights respectively. The final weights were obtained after a 24-hour starvation period on the last day of the experiment.

The stilbestrol treatment increased the *in vitro* glucose uptake and glycogen synthesis of the insulin stimulated diaphragm. Table 10 shows these data.

Both liver and diaphragm glycogen levels were determined immediately after sacrificing the animals. Approximately $\frac{1}{4}$ of the hemidiaphragm was used for this glycogen determination. The glycogen level in the liver was significantly increased. This increase was not at the expense of muscle glycogen since the diaphragm muscle glycogen was the same as the control. The results are given in Table 10.

DISCUSSION

The work of Griffiths and Young (21) and Lewis *et al.* (16) suggests that estrogens affect carbohydrate metabolism by means of a stimulatory effect on β cell secretion of insulin or on β cell regeneration. The results reported in this paper show that certain estrogenic substances administered

TABLE 9. THE EFFECT OF ONE SUBCUTANEOUS DOSE STILBESTROL (300 γ/100 GM.) EIGHTEEN HOURS PRIOR TO ASSESSING GLUCOSE UPTAKE AND GLYCOGEN DEPOSITION IN HEMIDIAPHRAGM AND GLYCOGEN IN LIVER

Results expressed as mg./g. tissue. Insulin added at 100 milliunits/ml. Experiment No. 152.

Measurement	Control		Stilbestrol	
	1 Unstim.	2 Insulin Stim.	3 Unstim.	4 Insulin Stim.
Glucose uptake	3.38	4.79	3.76	5.14
Insulin effect:	1.41		1.38	
	S.E. mean = ±0.18			
	t between 1 and 2 = 5.53‡ t between 3 and 4 = 5.43‡ t between 2 and 4 = N.S.			
Glycogen deposition	1.44	2.03	1.58	2.16
Insulin effect:	0.59		0.58	
	S.E. mean = ±0.14			
	t between 1 and 2 = 2.85† t between 3 and 4 = 2.80† t between 2 and 4 = N.S.			
Liver glycogen	0.34		2.42	
	S.E. mean = ±0.54			
	t between control and stilbestrol = 2.70*			

Note: Each value is the mean of 8 determinations.

* = $P < 0.05$.
† = $P < 0.01$.
‡ = $P < 0.001$.

for a short time *in vivo* can stimulate glucose utilization in isolated muscle. There is also a marked increase in the responsiveness of the added insulin. The mechanism of this potentiating effect can be inferred from the observed inhibition of adrenal corticoid secretion, gonad suppression and reduced somatic growth. Thus, it seems likely that the increased insulin sponsiveness of muscle arising from estrogen treatment is due, at least in part, to a decreased pituitary secretion of the diabetogenic substance. This may arise from an estrogen induced shift in pituitary secretions. The adrenal and gonad inhibiting properties of the estrogens might stimulate an increase in corticotropin and gonadotropin secretion at the expense of growth or diabetogenic hormone. A study of the effects of progestational compounds which inhibit mainly pituitary gonadotropin secretion may help to determine which pituitary tropin is concerned with diabetogenesis. Both natural and synthetic androgenic steroids will also be examined in this system to help in assessing these complex interactions and to give clues as to the functional groups involved. A recent paper by Meyer and Clifton (35) has shown in the case of long term stilbestrol treatment with subcutaneous pellets, that prolactin secretion was stimulated. The evidence

was also suggestive of increased corticotropin and decreased growth hormone secretion.

It is quite obvious from the experimental literature that many cases of diabetes are not the result of a simple lack of insulin but perhaps more a lack of utilizable insulin. Even so, the supplementation with extra insulin does not cure all the metabolic imbalances of many types of diabetes, especially those disturbances leading to vascular degeneration. A restoration to normal of the abnormal pituitary-adrenal function that must exist in many diabetic states might not only restore the effective use of endogenous insulin but mitigate the other degenerative changes. This might well be accomplished with inhibitors of pituitary growth hormone or diabetogenic hormone, either directly, or indirectly via target organ inhibition. In this regard it is interesting that a recent preliminary clinical report of a case of "brittle" diabetes treated with ethinyl estradiol led to a marked stabilization as well as significant lessening of the insulin requirements (36).

TABLE 10. EFFECT OF A 500 γ/100 GM. DAILY DOSE STILBESTROL FOR 14 DAYS ON GLUCOSE UPTAKE AND GLYCOGEN DEPOSITION IN HEMIDIAPHRAGM *in Vitro* AND ON LIVER AND DIAPHRAGM MUSCLE GLYCOGEN AT SACRIFICE

Results expressed as mg./gm. tissue. Insulin added at 100 milliunits/ml. Experiment No. 166.

Measurement	Control		Stilbestrol	
	1 Unstim.	2 Insulin stim.	3 Unstim.	4 Insulin stim.
Glucose uptake	3.49	4.45	4.11	6.16
Insulin effect:	0.96		1.05	
	S.E. mean = ±0.43			
	t between 1 and 2 = 1.57 N.S. t between 3 and 4 = 3.36† t between 2 and 4 = 2.80†			
Glycogen deposition	1.71	2.25	1.98	3.08
Insulin effect:	0.54		1.10	
	S.E. mean = ±0.11			
	t between 1 and 2 = 3.38† t between 3 and 4 = 6.88‡ t between 2 and 4 = 5.19‡			
Liver glycogen at sacrifice	0.40		1.11	
	S.E. mean = ±0.27			
	t between control and stilbestrol = 3.74†			
Diaphragm glycogen at sacrifice	0.70		0.91	
	S.E. mean = ±0.08			
	t between control and stilbestrol = 1.91 N.S.			

Note: Each value is the mean of 8 determinations.

† = P < 0.01.
‡ = P < 0.001.

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THE EFFECTS OF DIENCEPHALIC-MESENCEPHALIC LESIONS ON ALDOSTERONE AND HYDROCORTISONE SECRETION[1]

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ABSTRACT

Brain stem lesions (high frequency coagulation) were made in 35 large male cats; 19 served as unlesioned controls. Four hours after lesioning, secretion rates of hydrocortisone and aldosterone were determined by isolation of the steroids from adrenal venous blood by paper chromatographic methods. Transection of the midbrain reticular formation did not alter aldosterone or hydrocortisone secretion rates. Destruction of the ventral diencephalon significantly reduced hydrocortisone and aldosterone secretion. An even greater reduction in aldosterone levels was effected by lesions in the reticular formation of the midbrain and caudal diencephalon. These lesions likewise depressed the secretion of hydrocortisone. Secretion of aldosterone was significantly increased when the lesions extended caudally into the rostral half of the pons, without affecting hydrocortisone secretion.

INDIRECT evidence has accumulated from electrolyte balance studies and from observations of pathological states which supports the concept of a central nervous system mechanism for the control of the secretion of electrolyte-active steroids (1). Until recently, however, no direct experimental data relating the secretion of aldosterone to specific areas of the central nervous system have been available. The possibility of a diencephalic center for the regulation of aldosterone has been postulated. In the dog, decapitation or midcollicular decerebration with removal of the brain substance rostral to the section markedly diminished aldosterone secretion. On the other hand, decortication or section of the neural connections between the head and trunk were without effect (2). The present study was carried out in an effort to localize more precisely the area or areas which appear to be concerned in the regulation of the secretion of aldosterone by placing high frequency coagulation lesions in the brain stem and determining the effects on steroid secretion.

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METHODS

Fifty-four male cats (3–5 kg.) were included in this study, divided among the following groups: controls (14), controls given supportive therapy (5), transection through the central core of the midbrain (9), destruction of the ventral diencephalon (5) and destruction of areas in the central core of the caudal diencephalon and midbrain (21).

Operative Procedure. In each experiment the cat was anesthetized with ether followed by 1% chloralose (50 mg./kg.) intravenously. The trachea was cannulated. A femoral artery was cannulated for recording mean arterial blood pressure with a mercury manometer. The lesion was placed with the aid of a stereotaxic instrument and the animal allowed to recover without further anesthesia for a 4-hour period. During this time and throughout the remainder of the experiment care was taken to maintain blood pressure, body temperature and respiration within normal limits. When necessary, the arterial blood pressure was supported by the infusion of norepinephrine in 5% glucose. Body temperature was maintained with heat lamps when necessary. Alternating positive pressure respiration was routinely instituted to insure adequate oxygenation. Four hours after lesioning the left adrenal vein was ligated between the gland and the inferior vena cava and the lumbo-adrenal vein was cannulated. Heparin was injected intravenously to prevent clotting. Adrenal venous blood was collected over 2 to 3 hours. The blood was chilled during collection and frozen for later isolation of aldosterone and hydrocortisone.

In a control group of 14 intact cats the left lumbo-adrenal vein was cannulated and adrenal venous blood collected for 2 to 3 hours. In a second group of controls (5 cats) the same supportive measures (norepinephrine infusion, artificial respiration) were used as had been used in the lesioned animals, to rule out the possibility that these might have an effect on steroid secretion. A constant intravenous infusion of norepinephrine was given in dosages comparable to those required by the lesioned cats. The mean arterial blood pressure was prevented from rising above 100 mm. Hg by allowing the animal to bleed from the femoral artery into a reservoir elevated to a height equivalent to 100 mm. Hg (136 cm.). The animal was maintained in this state for the 4 hour equilibration period and the subsequent 2 to 3 hour collection period. Artificial respiration was used throughout these periods.

Placing of the Lesions. A Labtronics Stereotaxic Instrument was used. The calvarium was exposed and the bone removed above the area to be lesioned. An electrode $\frac{1}{2}$ mm. in diameter, insulated (Glyptal, G.E.) except for an exposed $\frac{1}{2}$ mm. tip, was inserted into the desired region. The Horsley-Clarke coordinates for lesions were obtained from an atlas of the cat diencephalon (3). The current from a high-frequency generator was passed through the steel electrode to an earth located on the head holder. This current, 4 megacycles frequency, 40 MA, for 30 seconds, produced a lesion 3mm. in diameter. Bilateral lesions of varying size were produced by repeated coagulation at adjacent sites.

The following Horsley-Clarke coordinates were used.

1) *Midbrain transection:* Frontal plane, +2 mm.; lateral, left and right, 1, 3 and 5 mm.; ventral 0, −2, −4, and −6 mm.

2) *Midbrain and supramammillary diencephalon:* Frontal plane, +5.0, 6.5, 8.0 and 9.5 mm.; lateral, left and right, 1, 3, and 5 mm.; ventral, −2 and −4 mm.

3) *Ventral diencephalon:* Frontal plane, +10, 12, 14 and 16 mm,; lateral, left and right, 1, 3 and 5 mm.; ventral, −2 and −5 mm.

At the end of the experiment the brains were fixed in 10% formalin and the block of tissue containing the lesion was removed and embedded in paraffin. Serial sections, 20 micra thick, were made and every twentieth section stained with cresyl violet. The extent of the lesion was determined both by gross and microscopic observation of the stained sections.

Isolation of Steroids from Adrenal Venous Blood. Hydrocortisone and aldosterone were isolated from the adrenal venous blood by paper chromatographic methods described in detail in earlier reports (2, 4). Aldosterone was isolated as the diacetate, using a micromethod. One-half inch paper strips were used for chromatography of the acetylated products instead of 1" strips. Elution of each cut was in 1.2 cc. methanol, permitting the use of cuvettes with a 1.5 cc. capacity. Quantitation was on the basis of absorption in the untraviolet, 240 mμ., $\epsilon = 15{,}850$. Mixed chromatograms with authentic aldosterone diacetate and bioassay were carried out to confirm the identity of the isolated aldosterone.

Statistical Analysis. The cats were arranged in the experimental groups, as presented, according to the location and extent of the lesion as determined by inspection of the prepared brain sections. The mean secretory rates were then determined from the individual values in each group. In some experiments the adrenal venous blood samples from two cats with similar lesions were pooled to permit more accurate quantitation of the steroid output. The secretory rate estimated from a pool of two cats had to be weighted when combined with values obtained from individual cats, in calculating the average for the experimental group. The significance of differences between means was calculated by standard statistical procedures. Each pooled sample contributed one degree of freedom.

RESULTS AND DISCUSSION

Control preparations

The average rate of secretion of aldosterone in the adrenal venous blood of 14 intact control cats was 47.9 ± 3.44* μg./100 kg. body weight/hr., and that of hydrocortisone, 24.8 ± 1.12 μg./kg. body weight/hr. (Table 1,A). In the five intact cats infused with norepinephrine and given artificial

* 1 standard error.

TABLE 1. RATES OF SECRETION OF ALDOSTERONE AND HYDROCORTISONE IN CONTROL GROUPS OF CATS

Cat No.	Aldosterone, μg./100 kg. body wt./hr.	Hydrocortisone, μg./kg. body wt./hr.
A. *Intact, no supportive therapy*		
39	64.5	23.5
40	39.3	25.8
42	31.4	21.8
61	54.6	26.4
63	52.6	20.76
82	66.5	29.1
49–51	55.5	21.05
50–52	37.5	27.6
53–54	47.5	21.6
62–64	40.3	29.5
	$\bar{x}$ 47.9	$\bar{x}$ 24.8
	S.E. 3.44	S.E. 1.12
B. *Intact, plus norepinephrine and artificial respiration*		
92	39.9	11.9
93	42.8	22.9
94	57.8	15.9
95	43.6	28.7
96	34.6	37.7
	$\bar{x}$ 43.74	$\bar{x}$ 23.42
	S.E. 3.85	S.E. 4.59

respiration (see Methods), the mean rate of aldosterone secretion was 43.74 ±3.85 μg./100 kg. body wt./hr., and that of hydrocortisone 23.42 ± 4.59 μg./kg. body wt./hr. (Table 1, B). Neither of these values is significantly different from the rates for intact controls ($P > .5$ for aldosterone, $P > .6$ for hydrocortisone). It appears from these results that the use of supportive measures does not influence the rates of secretion of the steroids.

Transection through the central core of the midbrain

The brain stem core was transected at the midbrain level in nine cats. The lesions extended from the superior colliculi at a point caudal to the posterior commissure ventrally through the central core of the midbrain into the cerebral peduncles at the rostral pons (Fig. 1). Laterally the lesions extended to each medial lemniscus at the level of the aqueduct. More ventrally the lateral extent of the lesions destroyed the ventral part of each medial lemniscus extending into the substantia nigra and cerebral peduncles. The structures transected in this central region in addition to those already mentioned included the mesencephalic reticular formation, the central tegmental tract, the medial longitudinal fasciculus, the nucleus of the third nerve, the red nucleus and the interpeduncular nucleus.

In this group of 9 cats, the aldosterone secretion rate was 45.0±4.2 μg./100 kg. body wt./hr., and hydrocortisone 21.21 ±2.81 μg./kg. body wt./hr. (Table 2). These values are not significantly different from those of the intact control group ($P > .6$ for aldosterone, $P > .2$ for hydrocortisone). This group may be considered as an *internal control* for the other experimental groups to be discussed. It appears that the placing of extensive central nervous system lesions, with the concomitant manipulation, surgery, and supportive therapy required, does not in itself alter the steroid secretion rate under the conditions of these experiments.

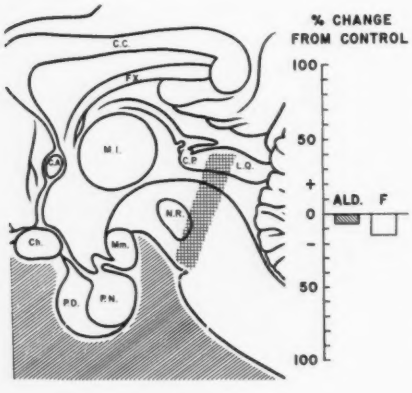

FIG. 1. *Transection Through the Central Core of the Midbrain* (9 cats).

The cross-hatched area is that including all lesions in this group. Aldosterone secretion was reduced by 6% from unlesioned control levels and hydrocortisone by 14.5%, as shown on the bar graph to the right of the figure.

Key to Figure: C.C., corpus callosum; FX., fornix; C.A., commissura anterior; C.P., commissura posterior; Ch., chiasma opticum; P.D., pars distalis; P.N., pars nervosa; Mm., Corpus mammillare; N.R. N. ruber; L.Q., lamina quadrigemina; M.I., massa intermedia.

TABLE 2. RATES OF SECRETION OF ALDOSTERONE AND HYDROCORTISONE IN CATS WITH TRANSECTION OF THE BRAIN STEM CORE AT THE MIDBRAIN LEVEL

Cat. No.	Aldosterone, μg./100 kg. body wt./hr.	Hydrocortisone, μg./kg. body wt./hr.
21	66.0	15.15
23	36.9	18.4
24	35.6	24.8
25	53.3	15.55
27	27.8	14.05
29	44.6	19.2
31	41.0	32.8
32	61.0	36.7
33	38.8	14.2
	$\bar{x}$ 45.0	$\bar{x}$ 21.21
	S.E. 4.2	S.E. 2.81

Sayers found that brain stem transection in the rat markedly reduces the release of ACTH in response to certain excitatory stimuli (5). Anderson et al. have similar findings in the dog (6). Had the release of ACTH been abolished in our transection experiments, the secretion rate of hydrocortisone would have been reduced. This was not the case. However, there are certain important differences between the experiments of Sayers and of Anderson and those reported here. The transections which were made in our experimental animals were largely limited to the central core of the brain stem, leaving the lateral ascending sensory paths essentially intact. The transections reported by Anderson and Sayers severed the entire brain stem. It seems likely, therefore, that the difference in results is related to the difference in the extent of midbrain transection.

Destruction of the ventral diencephalon

The lesions in these experiments destroyed the hypothalamus (Fig. 2). In cats 55 and 57 (Table 3) the lesion was confined to the hypothalamus from the anterior commissure to the supramammillary region. In cat 55

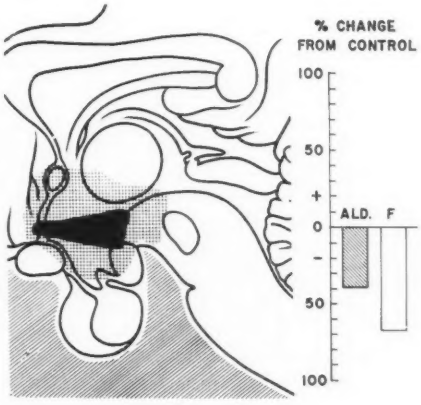

FIG. 2. *Destruction of the Ventral Diencephalon* (5 cats).

The cross-hatched area is that including all lesions in this group. The area common to all lesions in this group (solid black) is the hypothalamus. Hydrocortisone secretion was reduced by 67.8% from unlesioned control levels and aldosterone by 39.4%, as shown on the bar graph to the right of the figure.

TABLE 3. RATES OF SECRETION OF ALDOSTERONE AND HYDROCORTISONE IN CATS WITH LESIONS OF VENTRAL DIENCEPHALON

Cat. No.	Extent and variation of lesion	Aldosterone, μg./100 kg. body wt./hr.	Hydrocortisone, μg./kg. body wt./hr.
55	Hypothalamus, above optic chiasm to dorsal mammillary bodies	30.1	8.0
57	Hypothalamus	43.2	8.41
56–58	Hypothalamus and rostral half of midbrain	24.2	4.46
60	Hypothalamus and rostral half of midbrain	23.4	14.55
	$\bar{x}$	29.02	$\bar{x}$ 7.98
	S.E.	4.32	S.E. 2.13

the lesion was well above the optic chiasm and destroyed only the dorsal aspect of the mammillary bodies. The median eminence and vascular supply to the pituitary were apparently intact. In the other four cats in this group damage to the blood supply to the pituitary cannot be excluded. In addition, in cats 56, 58, and 60 the lesions extended into the rostral half of the midbrain. The secretion of hydrocortisone (Table 3) was significantly depressed (vs. intact controls, $P < .001$, vs. intact plus supportive therapy, $P < .05$, vs. brain stem transection, $P < .01$). This is in agreement with earlier reports that hypothalamic lesions depress ACTH release (7, 8, 9, 10).

Aldosterone secretion was also depressed (Table 3). The reduction in the secretion rate was statistically significant (vs. intact controls, $P < .02$, vs. intact plus supportive therapy, $P < .05$, vs. brain stem transection, $P < .05$). The reduction in aldosterone output is of the order of magnitude seen after hypophysectomy (11, 12), and is in accord with the concept that the regulation of aldosterone secretion may in part be via the pituitary. It is of interest, in this connection, that Daily and Ganong failed to find any effect upon urinary electrolyte excretion by lesions in this region (13), nor for that matter, is hypophysectomy followed by any marked change in electrolyte handling (14, 15). This suggests that rather profound changes in steroid output must occur before renal electrolyte handling is measurably affected.

Of the five animals in this group, the lowest rates of output of aldosterone were found in the three in which the lesion extended more caudally into the midbrain (cats 56, 58 and 60, Table 3). It was later found that lesions in the midbrain which spared the pituitary led to an even greater depression in aldosterone secretion rate (v.i.).

Destruction of the central core of the midbrain and caudal diencephalon

The central core of the brain stem throughout the midbrain and supramammillary diencephalon was destroyed in seven cats (Table 4, A). These lesions extended rostrally through the midbrain tegmentum from the level

of the midbrain transection described above into the supramammillary diencephalon (Fig. 3). The lesions extended from the central grey ventrally to the interpeduncular nucleus and laterally as shown in Figure 4. The structures destroyed in this lesion, in addition to those already described in the transection first mentioned, include almost the entire mesencephalic reticular formation, the zona incerta, and Forel's H_1 and H_2, as well as the following nuclei: Darkschewitsch, interstitial, posterior and lateral hypothalamic, subthalamic and nuclei in the caudal pole of the ventral thalamus.

In four of these seven cats (72, 75, 83, 74, Table 4, A) the lesion was found to be 1 to 2 mm. above the base of the midbrain. In the other three

TABLE 4. RATES OF SECRETION OF ALDOSTERONE AND HYDROCORTISONE IN CATS WITH LESIONS OF THE CENTRAL CORE OF THE MIDBRAIN AND CAUDAL DIENCEPHALON

Cat No.	Extent and variation of lesion	Aldosterone, μg./100 kg. body wt./hr.	Hydrocortisone, μg./kg. body wt./hr.
A. Central core midbrain and supramammillary diencephalon.			
72	Central core midbrain and supramammillary diencephalon, 1–2 mm. above base of midbrain	11.8	12.45
75	Same as above	21.7	4.67
83	Same as above	22.1	5.9
74	Same as above+dorsal ½ mammillary bodies	9.0	9.3
79	Central core midbrain and supramammillary diencephalon, extending to base of midbrain	11.0	6.65
43	Same as above+dorsal ½ mammillary bodies	15.5	9.03
78	Same as above	23.8	6.7
		$\bar{x}$ 16.4	$\bar{x}$ 7.81
		S.E. 2.3	S.E. 0.993
B. Central core midbrain lesions sparing or only partially involving the diencephalon.			
22	Central core midbrain	29.5	13.53
26	Central core midbrain	25.7	18.7
28	Central core midbrain	40.0	12.75
30	Central core midbrain	11.5	10.15
71	Midbrain tegmentum	16.5	14.4
73	Midbrain tegmentum+partial supramammillary diencephalon	4.2	13.5
76	Same as above but sparing one side of the central supramammillary region	24.3	15.83
80	Midbrain tegmentum+partial supramammillary diencephalon	38.6	15.0
44	Same as above but sparing the entire central supramammillary region	50.0	20.4
77	Same as above	42.6	25.43
		$\bar{x}$ 28.29	$\bar{x}$ 15.97
		S.E. 4.65	S.E. 1.4
C. Central core brain stem lesions involving the rostral pons.			
20	Central core supramammillary diencephalon caudal through midbrain into rostral half of pons	58.3	7.2
45	Same as above	83.6	29.0
46–47	Same as above	89.3	33 3
		$\bar{x}$ 80.1	$\bar{x}$ 25.7
		S.E. 9.07	S.E. 7.66

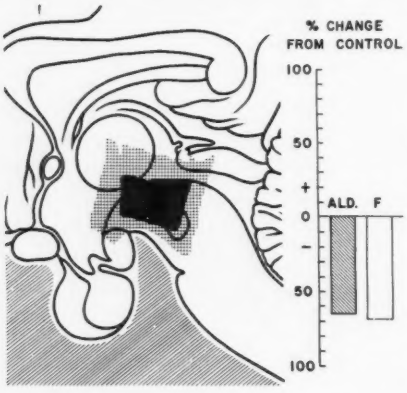

FIG. 3. *Destruction of the Central Core of the Midbrain and Supramammillary Diencephalon* (7 cats).

The cross-hatched area is that including all lesions in this group, the solid black that common to all the lesions. The bar graph shows that aldosterone secretion was reduced by 65.8% from unlesioned control levels and hydrocortisone by 68.5%.

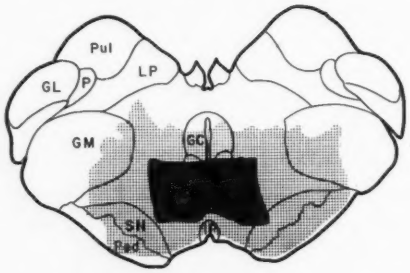

FIG. 4. *Transverse Section at the Level of the Habenular Nuclei.* (Lesion of the Central Core of the Midbrain and Supramammillary Diencephalon)

The cross-hatched area is that including all lesions in this group. The solid black area is that common to all lesions in this group.

Key to Figure: Pul., Pulvinar; L.P., N. lateralis posterior; P., N. posterior; G.L., corpus geniculatum laterale; G.M., corpus geniculatum mediale; S.N., Substantia nigra; Ped., Pendunculus cerebralis; G.C., griseum centrale; I.P., N. interpeduncularis.

(79, 43, 78) the lesion extended to the base, destroying the interpeduncular nucleus, the central tegmental nucleus of Tsai, and the mammillary peduncles. In cats 43, 78 and 74 the lesion also destroyed the dorsal one-half of the mammillary bodies.

It was of interest to determine the extent of pituitary damage in this lesioned group. In cats 72, 75, 83 and 79 the mammillary bodies were intact and the lesion ended 3–4 mm. above the pituitary fossa. For this reason it appeared unlikely that pituitary damage could have resulted. In cats 43 and 78 because of mammillary body involvement in the lesion the pituitary was examined histologically and appeared to be undamaged. Unfortunately, the pituitary was not examined in cat 74, in which mammillary damage was observed, and no statement as to the possible pituitary involvement can be made.

The effects of each of these lesions are presented in Table 4, A. In every instance the rates of secretion of aldosterone and hydrocortisone were reduced from the control level. The extent of reduction was somewhat variable: in some instances (cats 72, 74 and 79, Table 4, A) the aldosterone secretion rate was markedly depressed, while in others (cats 75, 83 and 78) the effect was not as marked. The same variability in response was seen in hydrocortisone secretion. Nevertheless, the group considered as a whole

demonstrates a highly significant reduction in the secretion of both steroids as compared with the intact controls, the intact controls plus supportive therapy or the midbrain transection group (in each comparison $P < .01$).

From these experiments it seems clear that structures in the central core of the midbrain and caudal diencephalon act to maintain hydrocortisone and aldosterone secretion at the levels seen in the control animals.

The mechanism whereby this stimulatory effect is exerted remains unknown. The reduction in hydrocortisone secretion undoubtedly reflects a decrease in ACTH release. There is increasing evidence for hypothalamic control of ACTH secretion (7, 8, 9, 10). It may well be that the level of the activity of the hypothalamic centers involved in ACTH release is maintained by structures in the midbrain reticular formation.

The effects on aldosterone secretion are similarly unexplained. A diencephalic center for the control of aldosterone secretion has been postulated (2). Whether the diminished aldosterone output in the experiments reported here is due to destruction of a reticular formation structure which tonically stimulates the postulated center, or whether the secreto-motor mechanism itself is destroyed is a matter for speculation. It is of considerable interest that the rate of output of aldosterone in the midbrain lesion cats was significantly lower ($P < .05$) than in those with hypothalamic lesions. This is taken as presumptive evidence that the postulated center for regulation of aldosterone secretion is probably not located in the hypothalamus.

Central core midbrain lesions sparing or only partially involving the diencephalon

On histological examination 14 cats were found to have midbrain lesions which differed from those described above. In 10 cats the lesions of the central core of the midbrain spared the diencephalon to varying extents (Table 4, B and Fig. 5). In cats 22, 26, 28, and 30, the lesions extended from the superior colliculi to the interpeduncular nucleus, but did not extend more rostrally than the posterior commissure dorsally nor the interpeduncular nucleus ventrally. In cats 71, 73, 76 and 80, the midbrain tegmentum was destroyed, but the lesions did not extend into the supramammillary diencephalon at all (cat 71) or did so only partially (cats 73, 76, 80). One side of the central supramammillary region was left intact in cat 76. In cats 44 and 77, the entire central supramammillary region was intact.

This group showed marked variation in steroid secretion rates. In certain instances (cats 30, 71 and 73, Table 4, B) the aldosterone output was markedly depressed. In the others the level of secretion of the steroid varied from moderately low to control levels. Hydrocortisone secretion in these animals showed much less variation, though being on the average

somewhat lower than in intact animals. There appeared to be no consistent correlation with the extent of the lesion nor was there correlation between the effects on the two steroids. However, it appears that structures important in regulation of steroid release are present in this area. Overall statistical evaluation of the data from this group indicates that depression of steroid output resulted. The aldosterone secretion rate was significantly lower than in the intact controls ($P < .01$) or in those with brain stem transection ($P < .02$). The hydrocortisone secretion rate was significantly lower than in intact animals ($P < .001$), but on the other hand, was significantly higher than in the previous group ($P < .001$).

The lesions extended more caudally and also somewhat more dorsally

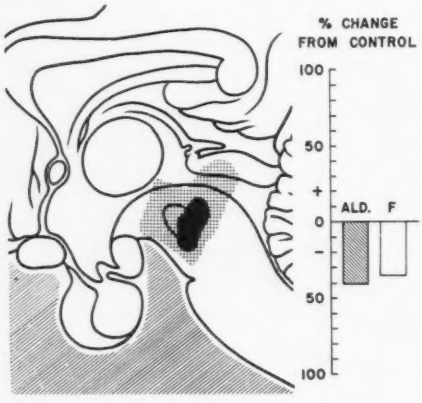

FIG. 5. *Central Core Midbrain Lesions Sparing or Only Partially Involving the Diencephalon* (10 cats)

The cross-hatched area is that including all lesions in this group, the solid black that common to all the lesions. The bar graph shows that aldosterone secretion was reduced by 40.9% from unlesioned control levels and hydrocortisone by 35.5%.

than those described in the previous group (cf. Fig. 3 and Fig. 5). The large size of the lesions make interpretation difficult, but a possible explanation for the variability in response of aldosterone is that *inhibitory* as well as stimulatory areas may have been involved in these lesions.

Support for this concept came from four additional cats in which surprisingly high rates of secretion of aldosterone were found (cats 20, 45, 46, 47, Table 4,C). The average level of aldosterone output was markedly increased over all the control groups. Although the series was small, the change was highly significant ($P < .01$ in every case). In these cats the most obvious variation in the extent of the central core midbrain and caudal diencephalon lesion was extension upward into the area caudal to the posterior commissure, as well as caudally into the tegmentum throughout the rostral half of the pons (Fig. 6). It seems likely that an area included in this lesion acts to inhibit aldosterone secretion; destruction of the area results in high levels of output of the steroid.

It should be emphasized that interpretation of the data in these experi-

ments is limited by the fact that the lesions involved large areas. Although the placing of such large lesions is of great value in locating areas of importance, it is clear that smaller, more discrete lesions in this effective region are necessary before more definitive interpretations can be made.

The ratio of aldosterone output to hydrocortisone output

Considerable evidence is available which indicates dissociation in the secretion of electrolyte-active and carbohydrate-active steroids (16). It was of considerable interest to calculate the ratio of secretion of the two steroids in the various lesions to determine whether such a dissociation could be demonstrated. In each experiment the rate of aldosterone secretion was related to that of hydrocortisone as the ratio, aldosterone, μg./

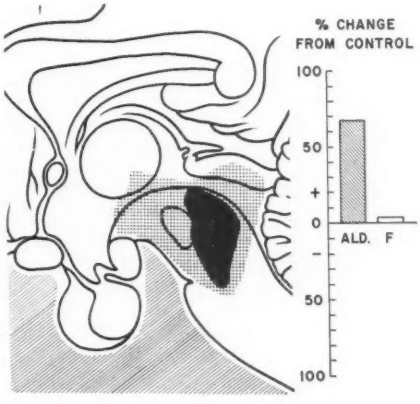

FIG. 6. *Central Core Brain Stem Lesions Involving the Rostral Pons* (4 cats)

The cross-hatched area is that including all the lesions in this group, the solid black that common to all the lesions. The bar graph shows that aldosterone secretion was increased by 67% above unlesioned control levels, while hydrocortisone secretion remained at control levels (3.5% above unlesioned controls).

100 kg. body wt./hr.: hydrocortisone, μg./kg. body wt./hr. The resulting ratios are summarized in Table 5.

For purposes of statistical evaluation the ratios from the intact controls, intact controls given supportive therapy and from the animals with central core midbrain transection were treated as a single control group (Pooled estimate from controls, Table 5). When compared with this ratio it was apparent that in two lesioned groups (lesions of the ventral diencephalon and lesions involving the rostral pons) the ratio of secretion of aldosterone to hydrocortisone was significantly altered ($P < .01$). In each instance the ratio, aldosterone/hydrocortisone was increased. In the lesions of the ventral diencephalon, this appeared to be due to a disproportionate decrease in hydrocortisone secretion (cf. Table 3), whereas in the lesions involving the rostral pons it was due to a disproportionate increase in aldosterone secretion (cf. Table 4, C). This finding lends further support to the concept that the output of the two steroids is independently controlled. On the

TABLE 5. RATIO OF SECRETION OF ALDOSTERONE TO HYDROCORTISONE IN UNLESIONED CATS AND IN CATS WITH LESIONS IN VARIOUS AREAS OF THE BRAIN STEM

Experimental group	Ratio, Aldosterone, μg./100 kg. body wt./hr. / Hydrocortisone, μg./kg. body wt./hr.
Intact Controls	1.98 ±0.176*
Intact Controls+Supportive Therapy	2.26 ±0.528
Transection of brain stem core at the midbrain level	2.34 ±0.336
Pooled estimate from Controls	*2.15 ±0.169*
Lesions of ventral diencephalon	4.27 ±0.849†
Lesions of central core midbrain and caudal diencephalon:	
a. Lesions of central core midbrain and caudal diencephalon	2.46 ±0.564
b. Lesions sparing or only partially involving the diencephalon	1.75 ±0.263
c. Lesions involving the rostral pons	4.06 ±1.61†

* Standard error.
† Statistically significant ($P < .01$) when compared with Pooled Estimate from Controls.

other hand, it is true that in general the lesions affected the secretion of the two steroids in the same direction. A possible explanation for this finding is that the reticular formation structures affecting the secretion of the steroids lie in overlapping regions, or that the lesions studied in these experiments were too large to distinguish closely related centers.

Vascular Effects of Brain Stem Lesions

In Table 6 are presented the mean blood pressures at the midpoint of the adrenal venous blood collection periods and the average adrenal blood flows in the various experiments. Since data on blood pressure and adrenal

TABLE 6. VASCULAR EFFECTS OF LESIONS OF THE BRAIN STEM

Experimental group	Mean B.P. mm. Hg.	Adrenal venous blood flow, cc./hr.	Adrenal resistance, mm. Hg./cc./hr.
Intact Controls	87 ±9.4*	43.5 ± 5.8	2.32 ± .41
Intact Controls+Supportive Therapy	100 ±0	28.7 ± 4.5	3.94 ± .72
Transection of brain stem core at the midbrain level	65.6 ±6.0†	48.8 ± 3.7†	1.46 ± .22†
Lesions of ventral diencephalon	75 ±2.5†	33.8 ± 7.7	2.67 ± 1.05
Lesions of central core midbrain and caudal diencephalon:			
a. Lesions of central core midbrain and caudal diencephalon	85 ±8.4	25.5 ± 8.1	4.30 ± .99
b. Lesions sparing or only partially involving the diencephalon	84.4 ±7.8	37.7 ± 5.7	2.91 ± .55
c. Lesions involving the rostral pons	80 ±0†	79.9 ±22†**	1.39 ± .71†

* Mean and standard error.
† Statistically significant ($P < .05$) when compared with values for the intact controls +supportive therapy.
** Statistically significant ($P < .05$) when compared with values for the intact control group.

flow were available, it seemed worthwhile to calculate adrenal resistance. The resistance values, expressed as mm. Hg/cc. flow/hr., are also presented in Table 6.

The blood pressure values in the lesioned groups were not significantly different from those in the intact control group in any instance. When the comparison was made between the lesioned groups and the intact controls plus supportive therapy, three groups were seen to have somewhat reduced mean blood pressure (transection of the central core of the midbrain, lesions of the ventral diencephalon and lesions involving the rostral pons, Table 6). In the first group, the aldosterone secretion rate was at the control level, in the second it was lower than in the controls, and in the third it was higher.

The adrenal bood flows did not vary significantly from the intact controls except in the group with lesions of the rostral pons. The adrenal blood flow in this group was higher than in the intact controls. When the adrenal blood flows were compared with those in the intact controls plus supportive therapy, it was found that in two groups, those with transection of the central core of the midbrain and with lesions involving the rostral pons, the average blood flows were significantly elevated.

It is of considerable interest that the increased adrenal flows in these instances were not a reflection of increased systemic blood pressure, but rather of reduced adrenal resistance (Table 6). However, the changes in adrenal resistance were not uniformly associated with changes in steroidogenesis.

Steroid secretion in the midbrain transection group was unchanged from that in the two control groups. The aldosterone secretion rate in the rostral pons group was greater than in the controls, but hydrocortisone secretion rate was not. The physiological significance of the decrease in adrenal resistance is not clear, although it may be related to involvement by the lesions of vasomotor pathways in the brain stem. That brain stem lesions induce alterations in hemodynamics is well known. However, in the absence of any correlation between steroidogenesis and adrenal hemodynamics, there appears to be no reason to believe that the changes in steroid output found in these experiments were mediated by variations in blood pressure or adrenal blood flow.

Acknowledgment

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We also wish to acknowledge a gift of norepinephrine from Dr. M. L. Tainter of the Sterling-Winthrop Research Institute.

Addendum

Since submission of this paper it has come to our attention that neuroanatomical evidence for pathways from mesencephalic areas to the diencephalon has been recently developed by Nauta and his coworkers. These findings are in excellent accord with our observations on the physiological effects of mesencephalic lesions. In experimental work carried out after submission of the manuscript the effects of a series of small lesions confined to the central gray substance and adjacent structures at the level of the posterior commissure have been determined. In four experiments out of seven significant reduction of aldosterone secretion was observed. Precise correlation of the extent of the reduction of aldosterone output with structures involved in the lesions has not as yet been possible. However, it seems evident that structures important in aldosterone regulation are present in this area.

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EFFECT OF TESTOSTERONE PROPIONATE ON THE APPETITE, BODY WEIGHT AND COMPOSITION OF THE NORMAL RAT[1]

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ABSTRACT

Testosterone propionate decreases the body weight of normal adult rats (Sprague-Dawley) in proportion to the dose administered (0.25–2.5 mg./day). The loss in body weight is due primarily to a loss of fat from the subcutaneous and abdominal areas with a concomitant calorically equivalent decrease in appetite. The protein content of the skin also is markedly decreased and a slight decrease is produced in the liver and carcass. The seminal vesicles and prostates and kidneys are increased in protein and fat but the net change for the animal is a slight decrease in total protein synthesis. Testosterone propionate decreases the rate of growth of young growing adult rats but not sufficiently to decrease the body weight. Cessation of androgen administration in both young and old normal rats results in an increase in appetite with a rapid restoration of body weight.

THE castrated rat fed *ad libitum* or a constant amount responds to androgen administration by an initial increase in the rate of growth followed by a decrease (1, 2). A similar but less marked response is produced in the normal rat on a constant food intake (3). In the course of other studies it was noted that the normal rat fed *ad libitum* was more responsive than the castrated rat to the inhibitory effect of testosterone propionate. This observation has been studied in detail.

PROCEDURE

The rats (Sprague-Dawley) were maintained in individual screen bottom cages in an air-conditioned room at 25.5–26.5° C. The artificial light was automatically regulated at 12 hours per day. The rats were fed *ad libitum* a diet consisting of parts per 100: Casein, 16.7; sucrose (granulated sugar), 20.4; corn starch (Argo), 40.7; hydrogenated vegetable oil (primex), 7.4; brewers' yeast (Fleischmann's 2019), 9.3; Wesson's salt mixture, 3.7; cellu-flour, 1.9. The average nitrogen content of the different batches of the diet was 31.0 mg./gm. Supplements of one drop of cod liver oil (Patch's) and one drop of a 34% mixed tocopherol concentrate[2] diluted tenfold with Wesson oil were given three times per week to each rat. The amount of food eaten was determined at 2-, 2- and 3-day intervals.

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[2] The tocopherol concentrate was kindly provided by Distillation Products, Inc., through the courtesy of Dr. P. L. Harris.

The urine and fecal collections and analyses for nitrogen were performed as previously described (2). The procedures for analyses of the tissues also have been described (1).

RESULTS

Effect of testosterone propionate[3] *on body weight and food intake of adult male rats:* In the first series of experiments (Fig. 1) the androgen produced a decrease in the rate of gain in body weight which apparently reached a maximum effect at about 1.0 mg./day. The inhibitory effect appeared after

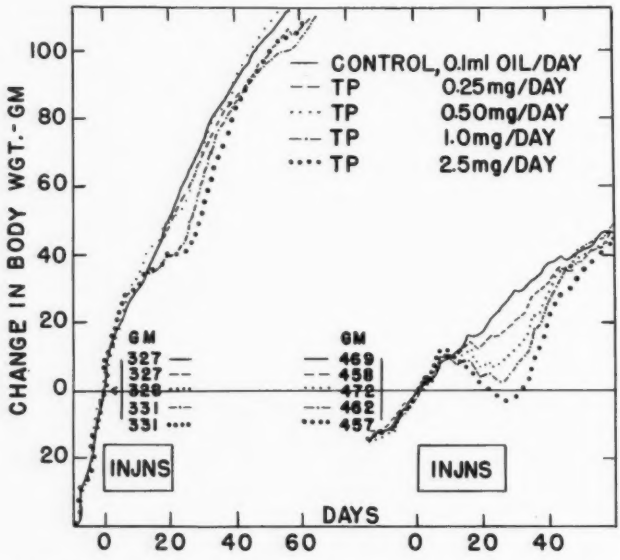

FIG. 1. Effect of testosterone propionate on the growth of normal male rats. The average body weights of each group of 10 rats at the beginning of the injection period are indicated along the 0 line.

about 8 days of injections. The higher dose of androgen produced a slight increase in the growth rate prior to the inhibitory effects. The food intake of the treated rats (Table 1) was slightly decreased at the three higher dose levels and was increased slightly for several weeks after cessation of injections.

On repeating the experiment with the same rats at a later date (Fig. 1), the effect of the androgen was much more marked. Now the rate of body weight gain not only was decreased but there was a sharp and steady loss in body weight for the duration of the injections. Furthermore, the maximum decrease was proportional to the log of the dose of testosterone propionate (Fig. 2). On cessation of the injections, a rapid restoration of the

[3] The testosterone propionate was generously provided as perandren (10 mg. and 5 mg./ml.) by Ciba Pharmaceutical Products, Inc.

TABLE 1. RELATION OF FOOD INTAKE OF ADULT MALE RATS TO DOSE OF TESTOSTERONE PROPIONATE[a]

Period[b]	Expt. I					Expt. II				
	Control, gm./day	TP mg./day				Control	TP mg./day			
		0.25	0.5	1.0	2.5		0.25	0.5	1.0	2.5
					Pre-injection					
1	18.6	18.7	19.4	19.3	18.7	18.3	18.1	18.7	18.2	18.4
2	21.0	20.0	20.8	19.7	20.7	17.5	17.4	18.4	17.3	18.0
					Injection					
3	19.9	18.4	20.2	19.6	19.7	17.9	17.4	18.3	17.6	18.0
4	19.2	18.8	18.6	18.4	18.3	18.3	17.6	17.9	16.8	16.9
5	18.9	18.6	19.0	18.0	18.0	18.0	17.1	17.1	16.4	16.0
6						18.2	17.4	17.6	16.6	16.8
					Post-injection					
7	18.9	19.1	19.7	19.0	18.7	18 4	17.9	18.7	18.2	18.2
8	18.6	19.0	19.7	19.6	19.0	18.3	17.7	18.9	18.7	19.6
9	18.6	18.9	19.8	19.4	19.7	18.2	17.6	18.9	18.3	19.3
10	18.7	18.6	19.6	18.9	19.7	18.0	18.2	18.9	18.4	18.7
11	18.3	18.6	19.4	18.2	19.0	19.2	18.7	19.7	18.0	19.2
12	18.3	18.4	19.1	18.3	18.4					
13	18.3	18.4	19.0	18.3	18.3					

[a] The values are the averages of the 10 rats per group.
[b] Each period represents 7 days.

lost body weight occurred and the rate of growth was restored. The food intake of the rats varied (Table 1) with the changes in body weight (Fig. 1). The appetite of the animals at the two highest dose levels decreased during the injection periods and increased on withdrawal of the androgen. The animals receiving the lowest dose of androgen showed no significant changes in appetite while those receiving 0.5 mg./day showed an intermediate response.

Two other experiments at 2.5 mg./day of testosterone propionate with rats weighing 450–500 gm. gave results similar to those in Experiment 2.

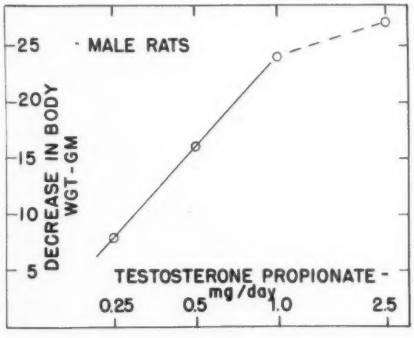

FIG. 2. Relationship of dose of testosterone propionate to the decrease in body weight of normal adult male rats. See Figure 1.

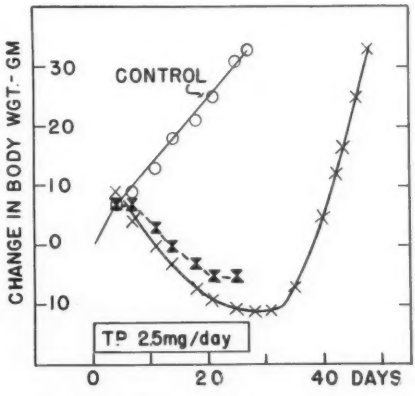

FIG. 3. Effect of testosterone propionate on the body weight of normal adult male rats.

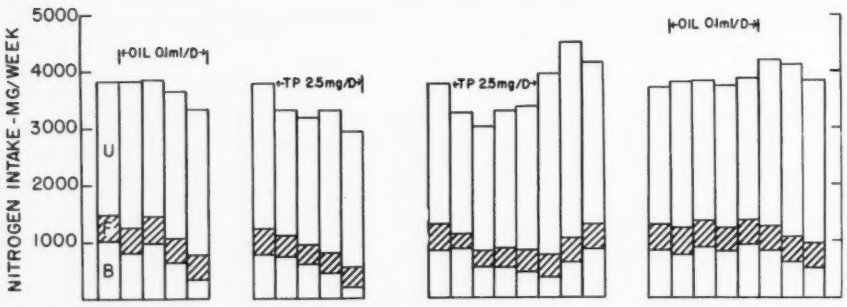

FIG. 4. The nitrogen balance of normal male rats treated with testosterone propionate. See Figure 3 and Table 5. Each bar represents a 7-day period. U = urine, F = feces and B = balance. The slightly lower nitrogen intake values in the last period of each group are attributable to removal of the food on the evening before the last morning collection prior to autopsy. The rats were fed *ad libitum*.

Furthermore, a group of castrated rats gave the same response as previously reported (1).

Effect of testosterone propionate on body weight and composition: In order to determine the nature of the change in body weight, the experiment at 2.5 mg./day in the older rats was repeated with an analysis of the excreta and of the bodies to determine which tissues and constituents were altered.

Body weight and food intake: The body weights (Fig. 3) and food intakes (Fig. 4) of the rats responded as in the previous study (Fig. 1). Furthermore, the positive nitrogen balance in the treated rats became slightly less than that in the control animals.

Body composition: The decrease in body weight was due to a decrease in fat (Table 2) which was restored by three weeks after cessation of the injections. The changes in body fat were calorically equivalent to the changes in food intake (Table 3). A small decrease in protein also occurred and this was not restored after cessation of injections. The decrease as observed by analyses of the tissues was comparable to that observed by nitrogen balance (Table 4).

The losses in weight and fat occurred about equally among the carcass,

TABLE 2. EFFECT OF TESTOSTERONE PROPIONATE (TP, 2.5 MG./DAY) ON THE BODY COMPOSITION OF THE NORMAL ADULT MALE RAT

Treatment	Rats No.	Body weight			H_2O, gm.	Protein, gm.	Fat, gm.	Rest, gm.
		Initial, gm.	At autopsy					
			Days	Gm.				
Control	8	455	25	467	276	87.0	79.0	25.1
TP-28	8	458	28	436	275	83.1	54.1	24.5
TP-28+(21)	9	453	49	469	275	83.6	81.6	28.9
Control	3	449	49	479	282	88.9	79.2	28.9

TABLE 3. COMPARISON OF THE CALORIC CHANGES IN BODY FAT AND FOOD INTAKE PRODUCED BY TESTOSTERONE PROPIONATE IN ADULT NORMAL RATS*

Period	Body fat		Food intake	
	Gm.	Cals.	Gm.	Cals.
Injection	−24.9	−227	−62	−248
Post-injection	+27.5	+250	+64	+256

* The calorie value of the food is 4.2 cals./gm. and a value of 9.1 cals./gm. is assumed for the body fat.

skin and hair and the viscera (Table 5). The fat changes were grossly visible. The carcass and skin and hair also showed a loss in both water and protein. These constituents except in the case of the carcass were restored on cessation of the injections. The seminal vesicles and prostates and the kidneys responded to the androgen with a gain in all of their constituents while the liver showed a small decrease. The composition of these organs was restored toward normal 21 days after androgen treatment was ended.

DISCUSSION

It is difficult to state whether the initial effect of the androgen is the inhibition of the appetite or the mobilization of body fat or whether these two phenomena are effected simultaneously. If the initial response to the androgen is an inhibition of appetite, then the mobilization of the body fat is in response to the restriction in caloric intake without a proportionate decrease in energy metabolism. The decrease in body weight, however, is apparent sooner than the inhibition of the appetite. In any event testosterone is able to stimulate a mobilization of body fat for energy purposes and at the same time proportionally inhibit the food intake. Thus, the fat depots are utilized without a compensatory replacement from dietary sources. The greater effectiveness of this phenomenon in the older animals may be due to the greater amount and different composition of the available body lipids. Comparable effects have been noted in castrated rats (1) and mice (4) but not to as great an extent as in the normal animal. These animals did not show a loss in body weight. Thus, the testis plays an interesting intermediary role in the ability of the rat to shift part of its energy needs from exogenous to endogenous sources. This property of the

TABLE 4. EFFECT OF TESTOSTERONE PROPIONATE (TP, 2.5 MG./DAY) ON THE NITROGEN BALANCE OF NORMAL MALE ADULT RATS FED *ad libitum*

Period	Days	Nitrogen retained/period					
		Control, mg.	T.P., mg.	Diff., mg.	Control, mg.	T.P., mg.	Diff., mg.
Basal	7	730	775	(+45)	844	860	+16
Injn.	28	2785	1975	−810	3449	2443	−1006
Post-injn.	21	—	—	—	1980	1873	−107
				−765			−1097

androgen may be a factor in the change in body fat which occurs with aging (5, 6).

The marked decrease of protein in the skin and the slight decrease in the liver and carcass accompanied by a simultaneous increase in that of the accessory sex organs and kidney are similar to those observed in the androgen-treated castrated rat (1). It is noteworthy that the greatest loss in protein occurred in the skin.

TABLE 5. EFFECT OF TESTOSTERONE PROPIONATE ON THE COMPOSITION OF THE TISSUES OF THE NORMAL ADULT MALE RAT[a]

	Carcass				Skin and hair			
	Wgt., gm.	H_2O, gm.	Prot., gm.	Fat, gm.	Wgt., gm.	H_2O, gm.	Prot., gm.	Fat, gm.
Control	273.1	179.7	52.1	22.6	108.3	49.4	25.7	30.8
TP-28	*263.1*	177.2	51.0	*16.3*	*92.3*	*46.6*	*22.7*	*21.2*
TP-28+(21)	268.6	173.8	50.4	25.6	111.9	52.3	24.2	30.6
Control	284.8	185.0	54.9	23.8	109.5	49.7	24.7	31.3
	Viscera				Liver			
Control	67.3	34.1	5.47	24.5	11.76	7.80	2.72	0.70
TP-28	*60.1*	35.8	5.39	*15.7*	*10.18*	*7.05*	*2.29*	*0.45*
TP-28+(21)	70.3	35.8	5.53	25.5	11.27	7.78	2.39	0.43
Control	67.3	35.2	5.78	23.3	11.87	8.14	2.58	0.46
	Kidney				Seminal Vesicle-Prostate			
Control	2.81	2.11	0.49	0.10	3.19	2.37	0.47	0.22
TP-28	*3.09*	2.32	0.55	0.11	*7.50*	*5.72*	*1.11*	*0.35*
TP-28+(21)	*3.06*	2.32	0.53	0.09	4.05	2.93	0.57	0.35
Control	2.76	2.06	0.50	0.09	3.13	2.28	0.47	0.24

[a] The values which showed changes are italicized.

The changes noted do not become very evident until doses above what may be considered a maximum physiological level (1, 2) are used. The slight effect on body weight noted at the lowest dose is similar to the numerous reports with similar or lower doses.

The response of the body weight to androgen administration has been previously observed to be affected by the age of the rat (1, 7) and it may be related to the amount of depot fat (1).

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EFFECTS OF ESTRADIOL-17-BETA-N-VALERATE ON ENDOSTEAL OSSIFICATION AND LINEAR GROWTH IN THE MOUSE FEMUR[1,2]

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ABSTRACT

Bone responses were studied in gonadectomized mice treated with amounts of estradiol-17-beta-n-valerate ranging from 0.1 to 20 μg.

Linear growth was accelerated more significantly in femurs from orchidectomized mice than those from ovariectomized mice.

Small amounts of the estrogen accelerated further linear growth of the femur, while at the same time they promoted the resorption of metaphyseal trabeculae.

Larger amounts of estradiol valerate inhibited resorption of metaphyseal trabeculae, and stimulated endosteal osteogenesis.

Some of the possible mechanisms underlying these observations are discussed.

INTRODUCTION

ESTROGENS stimulate endosteal ossification and cause the eventual sclerosis of epiphyseal cartilage (1, 2). In general large doses of esterified or non-esterified estrogens were given to obtain these results (3–6).

Esterification of estradiol-17-beta results in a prolongation of estrogenic effects upon target organs. Furthermore, estradiol-17-esters appear to further increase the durational action over estradiol-3-esters (7).

The primary consideration of the present work is to investigate the effects of different doses of estradiol valerate (Delestrogen®, Squibb[4]) on the mouses' femur. The comparative effects of this estrogenic steroid upon the pubic symphysis, vaginal mucosa, and uterus had already been reported (3).

MATERIALS AND METHODS

A total of 225 Brown Belt mice were studied. Of this number, 205 were castrated when 2–3 months old. One week after gonadectomy, they were injected once, biweekly, or once weekly for a four-week experimental period. They were divided into eight

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[2] Results read at the annual meeting of the American Association of Anatomists, Buffalo, N. Y., April 2–4, 1958.

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[4] The Delestrogen used in this investigation was received as a gift from E. R. Squibb & Sons, administered through Dr. E. C. Reifenstein, Jr., and Dr. J. T. Velardo.

groups and totals of 0.1, 0.5, 1.0, 5.0, 10.0, and 20.0 μg. of estradiol-17-beta-n-valerate were injected subcutaneously (Tables 1, 2). At autopsy the right femurs were removed and prepared for histological study by a method previously described (5). The left femurs were dried in an oven at 63° C for three days, after which they were cleaned, weighed, and measured. The dried bones were weighed on a Sartorius balance. The femur lengths were measured with a Helios vernier caliper from the head to the distal end of the condyles. The quantitative data were analyzed by using the unpaired *t* test for significance.

The histology of the distal end of the femurs were arbitrarily classified according to the characteristic of distal metaphyseal trabeculae which were present: no trabeculae (0), a few thin, short trabeculae (+), greater number of longer trabeculae (++), with

TABLE 1. FEMUR LENGTHS AND DRY WEIGHTS FROM MICE TREATED WITH ESTRADIOL-17-BETA-N-VALERATE

Treatment	Sex	No. of mice	Mean weight (mg.)*	Mean length (mm.)*
I. Intact Controls	♂	11	33.72 ± 3.94	13.78 ± .36
Intact Controls	♀	9	32.97 ± 2.92	13.74 ± .02
II. Castrate Controls	♂	12	33.78 ± 2.69	14.25 ± .38
Castrate Controls	♀	13	33.22 ± 4.01	13.83 ± .50
III. 0.1 μg. E.V.**	♂	12	34.53 ± 3.50	14.42 ± .08
0.1 μg. E.V.**	♀	10	34.43 ± 2.96	14.32 ± .32
IV. 0.5 μg. E.V.	♂	10	34.82 ± 2.30	14.24 ± .24
0.5 μg. E.V.	♀	20	32.30 ± 2.24	14.17 ± .23
V. 1.0 μg. E.V.	♂	11	34.41 ± 2.34	14.29 ± .28
1.0 μg. E.V.	♀	25	33.86 ± 2.80	14.08 ± .45
VI. 5.0 μg. E.V.	♂	10	35.12 ± 2.81	14.06 ± .17
5.0 μg. E.V.	♀	23	35.50 ± 2.87	14.08 ± .55
VII. 10.0 μg. E.V.	♂	11	37.36 ± 1.74	14.33 ± .52
10.0 μg. E.V.	♀	22	36.73 ± 2.88	14.26 ± .27
VIII. 20.0 μg. E.V.	♂	5	42.86 ± 1.21	14.60 ± .20
20.0 μg. E.V.	♀	21	36.75 ± 2.05	14.08 ± .37

* ± standard deviation.
** E.V. = Estradiol-17-beta-n-valerate.

some coalesced trabeculae (+++), coalesced trabeculae surrounding islands of red marrow which extended further into the diaphyses (++++). In order to eliminate some of the bias, slides were read and classified without knowing the treatment given. A minimum of two readings were made. Those classified differently during the first two readings were re-examined at a later date.

RESULTS

Femurs were similar in dry weight, length and histology in those mice given comparable total amounts of steroid. Therefore, the data were analyzed by pooling the samples according to the total injected amount of estradiol valerate (Tables 1, 2).

The mean dry weights of femurs from castrated and intact male mice were similar, likewise femurs from similarly treated female mice. Femurs

were longer in gonadectomized than in intact mice. The lengths of femurs from ovariectomized mice were not significantly different from intact female mice, while femurs from orchidectomized mice were significantly longer than those from intact male mice ($P < .05$). Metaphyseal trabeculae of over half of the femurs from intact mice of both sexes were classified as ++ or more, while a third or less of the castrated mice were similarly classified.

Dry weights of femurs from gonadectomized male and female mice treated with 0.1, 0.5 and 1.0 μg. of estradiol valerate were not significantly

TABLE 2. DISTAL METAPHYSEAL RESPONSES OF FEMURS FROM MICE TREATED WITH ESTRADIOL-17-BETA-N-VALERATE

Treatment	Sex	Total number of mice	Observed bone responses				
			0	+	++	+++	++++
I. Intact Controls	♂	11	0	3	7	1	0
Intact Controls	♀	9	0	3	6	0	0
II. Castrate Controls	♂	12	2	6	4	0	0
Castrate Controls	♀	13	0	11	1	1	0
III. 0.1 μg. E.V.*	♂	12	1	9	2	0	0
0.1 μg. E.V.*	♀	10	1	9	0	0	0
IV. 0.5 μg. E.V.	♂	10	1	7	2	0	0
0.5 μg. E.V.	♀	20	2	14	4	0	0
V. 1.0 μg. E.V.	♂	11	0	7	4	0	0
1.0 μg. E.V.	♀	25	5	11	8	0	1
VI. 5.0 μg. E.V.	♂	10	0	6	3	1	0
5.0 μg. E.V.	♀	23	0	0	13	10	0
VII. 10.0 μg. E.V.	♂	11	0	6	3	2	0
10.0 μg. E.V.	♀	22	0	0	12	9	1
VIII. 20.0 μg. E.V.	♂	5	0	0	3	1	1
20.0 μg. E.V.	♀	21	0	0	11	10	0

* E.V.= Estradiol-17-beta-n-valerate.

heavier than the castrate and intact controls. In the same treated groups, femurs were significantly longer in male mice treated with 0.1 μg. estrogenic steroid than the intact controls ($P < .001$), while femurs were significantly longer in female mice treated with 0.1 and 0.5 μg., when compared with the intact controls ($P < .02$ and $P < .05$ respectively). Histologically, femurs from male mice treated with 0.1 and 0.5 μg. of steroid had approximately a fifth with ++ metaphyseal responses, while with 1.0 μg. over a third were so classified. Apparently it took 1.0 μg. of estradiol valerate to induce an equilibrium between metaphyseal resorption and endosteal ossification, whereas 0.1–0.5 μg. seemed to accelerate resorption of metaphyseal trabeculae. With 0.5 μg., metaphyses were similar to the femurs of ovariectomized mice. With 1.0 μg., there were indications of en-

dosteal ossification, since more than a third had ++ or more metaphyseal responses.

Femurs were significantly heavier in male mice treated with 10.0 and 20.0 μg. of estradiol valerate when compared with castrated controls ($P < .01$ and $P < .001$ respectively). On the other hand, femur lengths were not significantly different between all of the steroid-treated groups. Histologically, metaphyses from orchidectomized mice treated with 5.0 μg. were similar to the castrated controls. A fifth of the group treated with 10.0 μg. had +++ metaphyseal responses. Large numbers of coalesced trabeculae were present in the femurs of mice treated with 20.0 μg.

Femurs from ovariectomized mice treated with 10.0 and 20.0 μg. were significantly heavier than femurs from castrated controls ($P < .01$ in both cases). There was no significant inhibition or acceleration of growth in length in the femurs of female mice treated with these higher doses, when compared to the castrated or 0.5 and 0.1 μg. treated groups. Endosteal ossification (+++ or ++++) was great in approximately a half of the femurs from female mice treated with 5 μg. or more of the estrogenic steroid.

DISCUSSION

The weights of femurs in both castrated and intact mice were similar. Linear growth was significantly accelerated in male mice in as short a period as five weeks after gonadectomy. Although there was some increase in femur length in female mice, this was not significant. The acceleration of linear growth after gonadectomy in males is well known (2). The relatively smaller size of females has been attributed to the inhibiting effects of estrogens upon the somatotrophic hormones (8). It appears that bones from ovariectomized mice were conditioned by the previously present estrogens not to respond as readily to linear growth after castration. This is supported by some indirect evidence. Female mice castrated at birth do not respond as readily to interpubic ligament formation when treated with estrogen and relaxin, as do mice ovariectomized after puberty and similarly treated (9). Metaphyseal trabeculae were more numerous and thicker in the intact mice than in the castrated ones. With subthreshold amounts of gonadal hormones present after gonadectomy, catabolic effects upon the metaphyses became manifested. The anabolic effects of gonadal hormones have been studied extensively, and have been used in the treatment of senile osteoporosis (10).

Femurs did not become significantly heavier than controls until 10.0 or more micrograms of estradiol valerate were injected into the mice. Histologically, however, the coalescence of trabeculae was observed after 5.0 μg. was injected in the female and 10.0 μg. in the male. This fact indicates an initial relative differential sensitivity of adult mice femurs to

estrogens. The slower temporal response of male over female mice to high doses of estrogenic hormones has been observed by other investigators (11–14).

Femurs from mice treated with 0.1 μg. estradiol valerate were significantly longer than controls in both sexes, although their weights did not significantly differ. It appears that low doses of estrogens apparently accelerate linear growth, while higher amounts inhibit such. Likewise, small doses of estrogenic steroid appeared to promote the resorption of metaphyseal trabeculae, while large amounts inhibited bone resorption and promoted osteogenesis. The latter effects of estrogens upon bone are well known (1, 2, 5, 6).

It is interesting to speculate that low doses of estrogens may act in concert with the somatotrophic hormone of the hypophysis and may exert synergistic actions upon the latter's action upon linear growth of bone. At the same time, these same low doses of estrogens may promote catabolic responses in the metaphyses along with the parathyroids, and thereby influence the feedback mechanism of calcium metabolism.

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SUPEROVULATION IN THE IMMATURE RAT AS A POSSIBLE ASSAY FOR LH AND HCG[1]

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ABSTRACT

It has been demonstrated that superovulation can be used as a quantitative assay for LH and HCG. The use of PMS to induce maximal growth and number of Graafian follicles results in an increase in the magnitude of the response. Treatment with a single dose of PMS failed to induce ovulation; however, injection of high dosages of PMS (10 to 20 I.U.) in immature rats pretreated with PMS caused 20 to 60% of the animals to ovulate. The intravenous injection of both HCG and LH increased the sensitivity of the assay by a factor of 10, as compared to the subcutaneous route of administration. A minimal response was obtained with 0.025 I.U. of HCG and 0.1 μg. of the Armour's standard LH given i.v. In the latter instance this test appeared to be 150-fold more sensitive than the test involving increase in weight of the prostate. A statistical analysis of the assay is given indicating a high precision for the average response. The λ_m for HCG and LH was 0.21 when given subcutaneously and 0.07 when given i.v. Preliminary tests with urine from normal males and females revealed the presence of an ovulation factor although consistent results were not obtained.

PROGRESS toward a better understanding of the physiology and biochemistry of the pituitary gonadotropins has been handicapped by the lack of a specific and sensitive assay for the presence of these substances in the urine. In view of the known synergism between the two gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), and the general contamination of one with the other in the presently available preparations, a specific response is of paramount importance. While a great deal of work has been done on the determination of gonadotropin in human urine (1), it is obvious that this is an assay of total gonadotropic activity and not of a single entity. Recently, McArthur (2) has employed the ventral prostate of the hypophysectomized rat as an indicator for ICSH (LH). This again is open to the problem of synergism with FSH or possible interference from prolactin (3).

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A second possible approach to the assay of LH has been the ovulatory response to gonadotropins, as originally reported by Smith and Engle (4) and used for the detection of HCG and pituitary gonadotropin (5 through 12). In spite of its sporadic use over a number of years the technique has not received much attention and has in general been discounted by most investigators.

The present investigation was undertaken to determine the feasibility of using the ovulatory response for an LH assay, i.e. to determine whether a quantitative relationship existed between the amount of human chorionic gonadotropin (HCG) or LH injected and the number of ova released. Evidence will be presented indicating the excellent sensitivity obtained by using immature female rats pretreated with pregnant mare serum gonadotropin (PMS) and followed by intravenous injection of LH or HCG. The problems of biologic variation, precision of assay, and dose-response relationship will be discussed.

MATERIALS AND METHODS

Approximately 500 female rats from the Charles River Laboratories were used in this investigation. The animals were obtained at 18 to 19 days of age and treatment was started at 21 days of age. The animals were housed in air conditioned rooms in groups of 5 and whenever possible each group received identical treatment.

All hormones were injected subcutaneously or intravenously (i.v.) in an aqueous medium. Either physiological saline or phosphate buffer at pH 8 was used. The urines were prepared either by the kaolin adsorption technique (13) or the alcohol precipitation method (14) and stored as a dry powder. The animals were injected with a priming dose of 30 I.U. of PMS,[4] followed by an injection of the ovulating substances 56 hours later (15). Purified preparations of HCG[4] and LH,[5] partially purified preparations of human urine, and crude extracts of rat pituitary were used as sources of ovulating hormone or hormones.

In the initial investigation, rats were killed at various time intervals after injection of the ovulating substance in order to determine the time of maximum response. Thereafter, all animals were killed at 18 to 24 hours after the final injection (15). At autopsy the Fallopian tubes were removed and examined under a dissecting microscope at a magnification of 40×. Ovulation was easily noted by the presence of an enlarged translucent segment of the tube, through which the ova could be seen (16). The segment was then punctured with a dissecting needle and the entire cumulus clot containing the ova was expelled in a single mass. The egg mass was treated with hyaluronidase or spread out with dissecting needles and the number of ova counted. This was found to be necessary due to the large number of eggs released at ovulation.

RESULTS

Time for Ovulation to Occur

The initial experiment was designed to determine the time at which the ovulating hormone should be injected following the PMS in order to get

[4] The PMS and HCG (APL^{Rx}) were obtained through the courtesy of Ayerst Laboratories, N.Y.C.

[5] The LH and FSH were obtained through the courtesy of Dr. Steelman, Armour Laboratories, Chicago.

maximal ovulation. HCG was used as the ovulating hormone in this series of experiments and PMS was used to promote growth of the follicles. The animals were injected with 30 I.U. of PMS and 56 hours later were injected with the HCG. The animals were killed at various intervals after the injection of HCG and the number of ova released and percentage of rats ovulating recorded (Fig. 1). It is apparent that minimal ovulation appeared in 8 hours and was complete by 17 hours after injection of the HCG. A maximum of 31 ova per rat was obtained at 17 hours when 100% ovulation was noted, and this value remained unchanged for the additional 8 hours studied in this experiment. It was noted that the egg clot was beginning to disintegrate towards the end of the period but the ova were still loosely clumped and could be easily removed without any loss. From this experiment it was concluded that the optimal time for detection of ova in the fallopian tube was from 18 to 24 hours after the injection of the ovulating hormone and this schedule was used in all subsequent studies.

Ovulation Following HCG

A good dose-response releationship may be seen for either the per cent rats ovulating (Table 1) or the average number of ova released following the subcutaneous injection of HCG (Fig. 2). The number of eggs increased from a mean of 0.5 at 0.5 I.U. of HCG to a maximum of 51.8 at 20 I.U. The

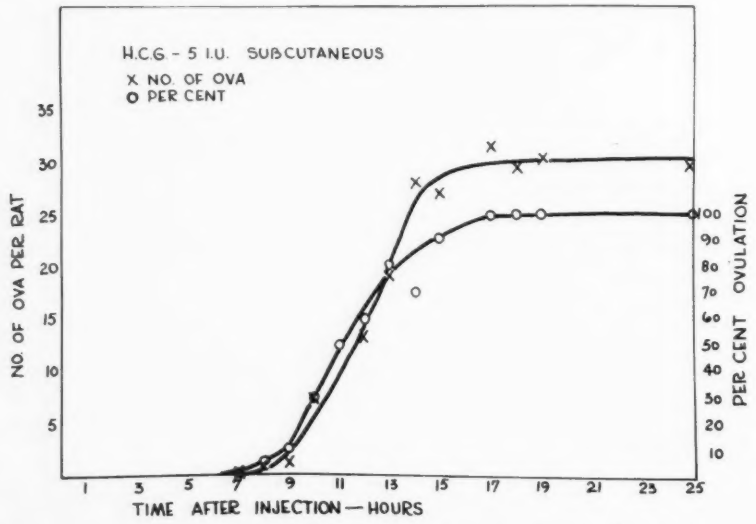

FIG. 1. The percentage of immature rats ovulating and the number of ova discharged as a function of time following the subcutaneous injection of 5 I.U. of HCG in immature rats primed with a single injection of 30 I.U. of PMS. Zero time is 56 hours after the injection of PMS and the time at which HCG was injected.

TABLE 1. EFFECT OF HCG AND LH ON THE INCIDENCE OF OVULATION IN THE IMMATURE RAT PRETREATED WITH 30 I.U. OF PMS

Treatment	No. of rats	Dose	% rats ovulating
	14	0.5 I.U.	7.1
	20	1.0 I.U.	35.0
	22	2.0 I.U.	45.4
HCG subcut.	20	5.0 I.U.	100.0
	16	10.0 I.U.	100.0
	10	20.0 I.U.	100.0
	15	0.025 I.U.	13.3
	24	0.05 I.U.	37.5
	16	0.1 I.U.	43.7
	18	0.2 I.U.	67.0
HCG intraven.	25	0.5 I.U.	88.0
	20	1.0 I.U.	100.0
	15	2.0 I.U.	100.0
	15	10.0 I.U.	100.0
	5	1.0 μg.	0.0
	10	2.0 μg.	40.0
LH subcut.	10	5.0 μg.	70.0
	8	10.0 μg.	100.0
	7	20.0 μg.	100.0
	10	0.05 μg.	20.0
	20	0.1 μg.	35.0
	19	0.2 μg.	47.3
	10	0.5 μg.	50.0
LH intraven.	15	1.0 μg.	100.0
	12	2.0 μg.	100.0
	15	10.0 μg.	100.0
	10	20.0 μg.	100.0
	12	100.0 μg.	100.0

slope for the response based on the per cent of rats ovulating was steeper than that noted for the number of ova, and a 100% response was obtained with 5. I.U. of HCG at which point the rats had ovulated on the average only 29.2 ova, as compared with the maximum of 51.8 ova.

The intravenous administration of HCG also gave a good dose-response relationship for both the per cent animals ovulating (Table 1) and the average number of ova released (Fig. 3). Again the 100% ovulating response was reached prior to the release of the maximum number of eggs. This was not as marked in the intravenously treated rats as in the subcutaneously injected ones. The intravenous route caused a 10 to 15-fold increase in the sensitivity of the reponse.

Ovulation Following LH

Figures 4 and 5 contain the data on the number of ova released following the subcutaneous and intravenous injections of LH, respectively, while the corresponding data on the per cent of rats ovulating are recorded in Table 1. Again a dose-response relationship is obtained with both end points and with both routes of administration. A steeper response was obtained with regard to the number of ova released following the subcutaneous route

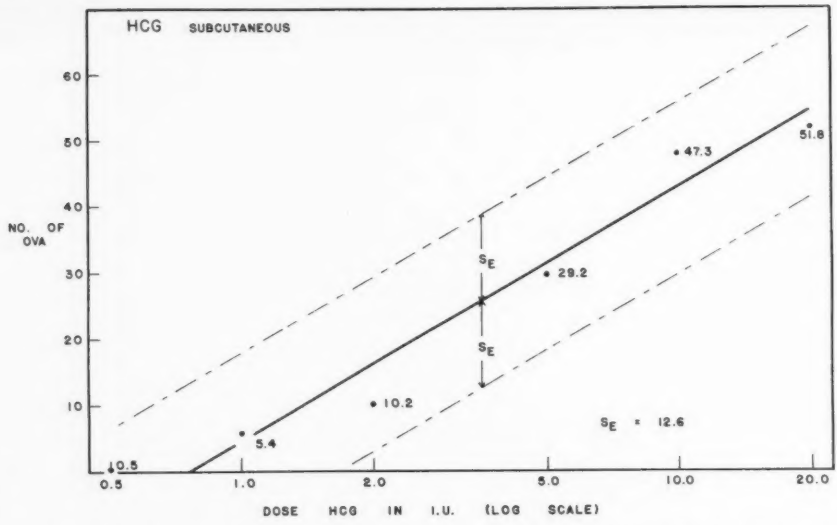

FIG. 2. Log-dose vs. response relationship for the average number of ova released as a function of HCG injected subcutaneously in immature rats primed with 30 I.U. of PMS. Mean number of ova is indicated at each dose. Standard error of estimate (S_E) is indicated by the interrupted line.

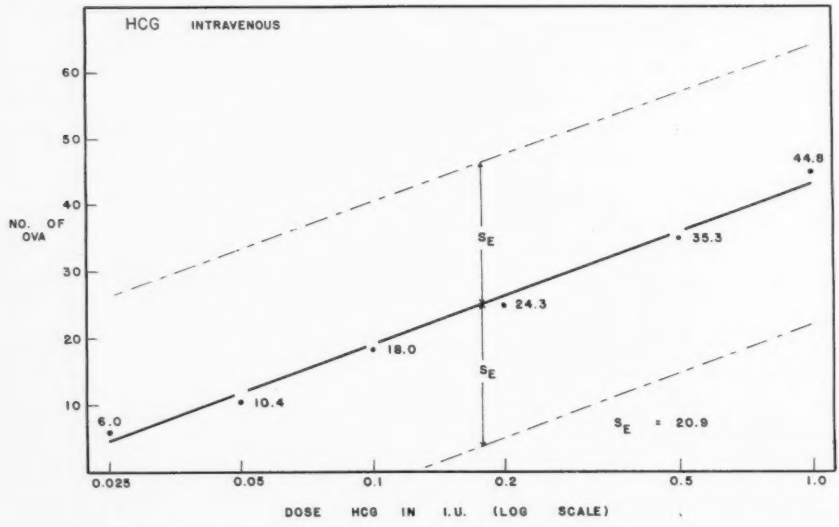

FIG. 3. Log-dose vs. response relationship for the average number of ova released as a function of HCG injected intravenously in immature rats primed with 30 I.U. of PMS. Mean number of ova is indicated at each dose. Standard error of estimate (S_E) is indicated by the interrupted line.

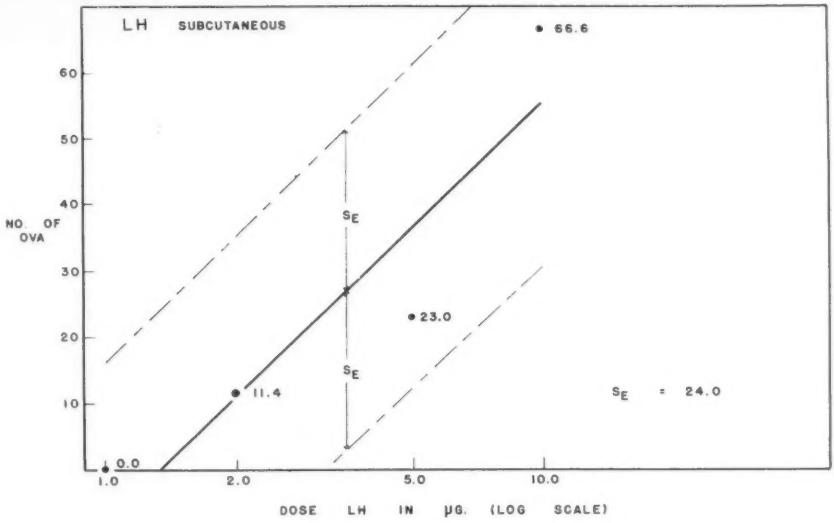

FIG. 4. Log-dose vs. response relationship for the average number of ova released as a function of LH injected subcutaneously in immature rats primed with 30 I.U. of PMS. Mean number of ova is indicated at each dose. Standard error of estimate (S_E) is indicated by the interrupted line.

as compared to the intravenous but the latter route increased the sensitivity by a factor of approximately ten. The curve for the number of ova plateaued with a dosage of 2 µg. of LH at a response to 59.2 ova. A further increase in LH to 100 µg. caused a release of an average of 72.6 ova; this represents a 22% increase in the average number of ova following a 20-fold increase in the LH dosage. Individual rats released as high as 105 ova in several instances.

Statistical Analysis of Data

All groups were tested for homogeneity of variance using Bartlett's test (17). No significant difference could be found at the alpha level of 0.9995, indicating homogeneous variances. The analysis of variance in each group showed that the sample means were significantly different at the 0.01 alpha level for all groups.[6] The regression lines for log-dose vs. response (Figs. 2, 3, 4, 5) were calculated by the method of least squares, and both the correlation coefficient, r, and the index of precision, λ, were determined for

[6] The term "group" is used to indicate all the rats receiving a given ovulating hormone by a given route of administration, i.e. all rats treated with HCG subcutaneously. The term "sample" is used to indicate all animals treated with a *single dose* of a given ovulating hormone by a single route of administration, i.e. 1.0 I.U. of HCG subcutaneously.

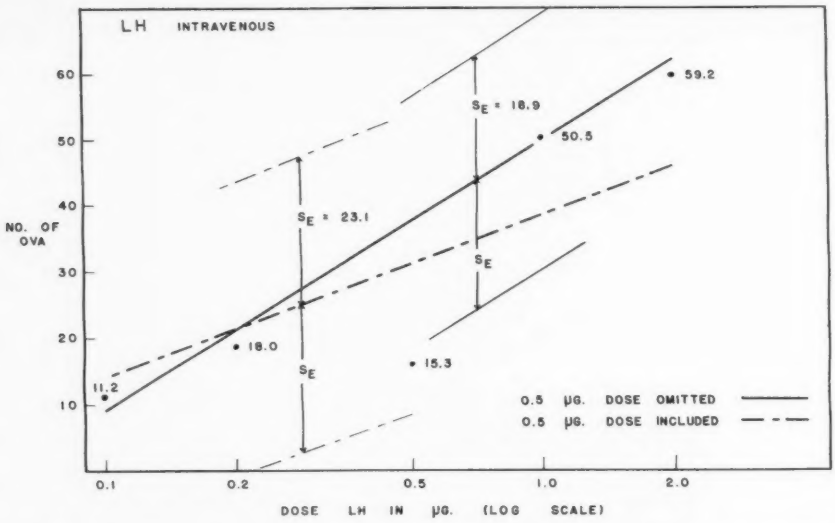

FIG. 5. Log-dose vs. response relationship for the average number of ova released as a function of LH injected intravenously in immature rats primed with 30 I.U. of PMS. Mean number of ova is indicated at each dose. The heavy solid line excludes the response to 0.5 µg. of LH and the heavy interrupted line includes all the data (see text). The light solid line and light interrupted lines represent the respective standard errors of estimate (S_E).

each group (Table 2). In addition, the standard error of estimate, S_E, was calculated in each instance and used to set confidence limits around the regression line. The calculations of r, S_E, and λ were made as follows:

X = the log of the dose given each animal
Y = the response of each animal
N = the number of animals in the group
SX = the sum of all the X's, i.e. the sum of the values obtained by multiplying the log of the dose for each sample by the number of animals in that sample.

TABLE 2. STATISTICAL ANALYSIS OF THE OVULATORY RESPONSE TO HCG AND LH IN THE IMMATURE RAT PRIMED WITH PMS

Treatment	Total animals	Dose range	r	λ	r_m	λ_m
HCG subcut	102	0.5 –20 I.U.	0.84	0.35	0.98	0.21
HCG iv	118	0.025– 1 I.U.	0.55	0.85	0.99	0.06
LH subcut.	33	1.0 –10 µg.	0.65	0.44	0.93	0.21
LH iv	72	0.1 – 2.0 µg.	0.73 (0.52)[1]	0.47 (0.81)[1]	0.99 (0.90)[1]	0.07 (0.46)[1]

[1] The first value given was calculated omitting the 0.5 µg. dose level, while the value in parenthesis was calculated including this dose level (see text).

SY = the sum of all the Y's.

SX^2 = the sum of squares of all the X's, i.e. the sum of the values obtained by multiplying the square of the log of the dose for each sample by the number of animals in that sample.

SY^2 = the sum of squares of all the Y's.

SXY = the sum of the products obtained by multiplying the response of each animal by the log of the dose given that animal.

$$r = \frac{SXY - \frac{(SX)(SY)}{N}}{\sqrt{\left[SX^2 - \frac{(SX)^2}{N}\right]\left[SY^2 \frac{(SY)^2}{N}\right]}}$$

$$S_E = \sqrt{\frac{\left[SY^2 - \frac{(SY)^2}{N}\right][1 - r^2]}{N - 2}}$$

$$\lambda = \frac{\sqrt{s^2}}{b}$$

s^2 is the pooled estimate of variance determined in Bartlett's test and b is the slope of the regression line.

Mather (18) stated "As the variance of any distribution decreases, the chances of finding a deviation from the mean of any given magnitude decreases too. In other words, the precision of our knowledge of the distribution has increased and we can predict the behavior of a single observation with greater accuracy." Since λ as calculated above is a function of the variance it is an index of the precision with which we can make predictions of the behavior of individual responses. These same considerations also apply to the values of r and S_E. For the purposes of a bioassay these statistics may be somewhat misleading, since, in an assay, one desires to make estimations based on sample means rather than on the responses of individual animals.

In order to obtain statistics which would indicate what can be expected of estimations based on sample means, statistics similar to those above but based on sample means alone were calculated and designated r_m, S_{E_m}, and λ_m. These calculations were made as follows:

$\overline{X}$ = the mean of the log of the dose for each sample (in this case $\overline{X} = X$).

$\overline{Y}$ = the mean response for each sample.

k = the number of samples in the group.

$S\overline{X}$ = the sum of all the $\overline{X}$'s in the group.

$S\overline{Y}$ = the sum of all the $\overline{Y}$'s in the group.

$S\overline{X}^2$ = the sum of squares of the $\overline{X}$'s.

$S\overline{Y}^2$ = the sum of squares of the $\overline{Y}$'s.

$S\overline{X}\,\overline{Y}$ = the sum of the products obtained by multiplying the $\overline{X}$ for each sample by its corresponding $\overline{Y}$.

r_m and S_{E_m} were calculated by substituting the $\overline{X}$ and $\overline{Y}$ notations above for the corresponding X and Y notations and k for N in the appropriate equations shown previously.

$$\lambda_m = \frac{S_{E_m}}{b}$$

The values of r_m calculated (Table 2) indicate that the sample means in each group are fitted well by a straight line, while the λ_m values indicate that estimations based on sample means can be made with high precision. The S_E values point out that fairly large numbers of animals are needed in each sample to obtain reliable means.

Two lines were fitted to the plot of the data for the group treated with LH intravenously (Fig. 5). The solid line was fitted after omitting the 0.5 μg. dose level, while the broken line was fitted including this dose level. Note that by omitting the 0.5 μg. dose level a line is obtained which fits the other four means in this group much more closely, comparable to the data obtained in the other three groups. The possibility has, therefore, been considered that some error was encountered at the 0.5 μg. dose level that was not encountered at any of the other dosages. The statistics for both the line which includes the 0.5 μg. dose level and the line which excludes it are recorded in Table 2. In plotting and analyzing the data for the group treated subcutaneously with LH (Fig. 4) the zero response at the 1.0 μg dose level was used since it was felt that this point lies very close to the threshold.

The λ_m values in Table 2 indicate that better precision is obtained with either HCG or LH administered intravenously. Intravenous injection also yields a more sensitive determination with either substance, as indicated by the dosage ranges. In the group treated with LH subcutaneously the number of animals used (Table 2) was considerably smaller than in the other groups. Comparison of the λ_m obtained with this group with the λ_m from the group treated with HCG subcutaneously shows that the smaller number of animals had no effect on the precision based on sample means. Comparison of the corresponding λ values indicates that possibly higher precision based on individual animals could be obtained with a larger number of animals. Fifteen animals at each dose level would probably yield satisfactory results in any of the groups.

DISCUSSION

While the ovulatory response has been used successfully for the determination of HCG no attempt has been made to compare the two majo

hormones that possess an ovulating activity and to quantify the reaction. It is apparent that a definite relationship exists between the amount of LH or HCG used and the number of ova released. Since it has already been shown (15) that PMS also determines the number of ova shed it would seem that the degree of ovulation is controlled by both the FSH and LH principles; the first hormone controlling the number of ova that can be released and the second hormone determining how many of the mature follicles can be ruptured. This would agree closely with the established concept of ovulation in the mammal (19).

The assay showed a high degree of sensitivity which was enhanced by the use of the intravenous route. In both instances, i.e. for HCG and LH, the intravenous administration increased the sensitivity by a factor of approximately 10. Under these conditions it is possible to detect as little as 0.1 I.U. of HCG as compared with 0.6 I.U. in the pregnant mouse (20), 2 to 25 I.U. in the male frog (21), 5 I.U. in the Friedman test, 10 I.U. in the rat hyperemia test (22), and 30 to 60 I.U. in the Weaver Finch Test (23). It would appear that the ovulatory response is one of the more sensitive reactions of HCG and that a follicle pretreated with PMS is extremely sensitive to the ovulating hormone. Since the work of Ladman and his colleagues (11, 20) involved a similar reaction but without superovulation the above statement is confirmed. It is probable that increased sensitivity can be obtained with the use of mice if only due to the dilution factor of decreased body size. Work is in progress along these lines.

Similar results to HCG were also obtained with LH. Again the curves for the two responses, i.e. per cent animals ovulating and number of ova released, depict good dose-response relationships and again the intravenous route increased the sensitivity by a factor of 10. Since there are no standard preparations for LH it is most difficult to make any sensitivity comparisons. Some ovulation was obtained with as little as 0.05 μg. of the LH preparation used in this study. Steelman (24) has indicated that 15 μg. of the same LH used in these experiments would cause a 100% increase in the weight of the prostate gland of the hypophysectomized rat. On the basis of these results the present test would appear to be approximately 150 times more sensitive. Preliminary analysis of several urine samples revealed the presence of an ovulating substance in the urine of men and cycling women. The results were not consistent, however, and more work is needed before the assay can be applied to extracts of human urine. Similarly, activity was also observed in crude extracts of the whole pituitary gland of rats.

In view of the lack of pure preparations of FSH and LH, no specificity tests were carried out. It was noted, however, that priming with PMS alone failed to induce significant ovulation in the immature rat. Only one instance of ovulation was noted in more than 200 rats treated with the single priming dose of PMS. However, contrary to the report of Rowlands (15) but in

TABLE 3. OVULATION FOLLOWING PMS IN THE IMMATURE RAT PRETREATED WITH 30 I.U. OF PMS

No. of rats	Body wt., gm.	Ovulating dose of PMS, I.U.	No. of ova per rat	% rats ovulating
5	49.0	10	11.4	20
5	50.4	20	18.2	60

agreement with the results of Cartland and Nelson (25), ovulation was obtained with PMS in rats previously primed with PMS (Table 3). This could simply indicate the presence of LH in the PMS preparation or the presence of an ovulatory property in the PMS molecule.

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UPTAKE OF RADIOSULFATE IN GROWING BONES OF COCKERELS TREATED WITH CORTISONE AND CERTAIN ANABOLIC-ANDROGENIC STEROIDS

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ABSTRACT

The effect of cortisone and certain androgenic anabolic steroids on the radiosulfate uptake by the three long bones of the young cockerel's leg, was investigated. Cortisone diminished and 17-ethyl-19-nortestosterone increased the capacity of growing bones to bind labelled sulfate. This was interpreted as a manifestation of the anti-anabolic and anabolic effect of these hormones respectively, on the synthesis of certain mucopolysaccharides of growing connective tissue, which specifically incorporate radiosulfate.

The anabolic steroid 17-ethyl-19-nortestosterone counteracted the anti-anabolic effect of cortisone on growing bone, when both hormones were administered simultaneously. Neither methyl testosterone nor testosterone propionate showed any significant effect on S^{35} uptake by the studied bones, nor did they counteract the anti-anabolic effect of cortisone.

CORTISONE is known to exhibit anti-anabolic effects both in birds and mammals (1, 2, 3, 4). Recently Hublé has shown that chondrogenesis in the proliferation zones of the proximal and distal ends of the long bones of the cockerel's leg was inhibited by cortisone treatment (4). This author was studying the histological changes induced in cartilage and bone by hormonal treatment.

In a series of experiments we were able to demonstrate that the catabolic and anabolic changes in cartilage and bone of rat may be studied satisfactorily with S^{35} labelled sulfate uptake procedure (5, 6, 7, 8, 9). It is known that the selective deposition of radiosulfate in certain tissues is due to the utilization of sulfate ions in the synthesis of chondroitin sulfate of the connective tissue ground substance and in the collagen (10, 11).

It was shown in our laboratory, that S^{35} uptake by the healing fractured bone in rats may be significantly inhibited by cortisone (7, 8) and promoted by certain anabolic steroids (5, 6, 7, 8, 9). This was interpreted to indicate that cortisone inhibited and anabolic steroids stimulated the production of certain mucopolysaccharides which are essential in collagen fibrillogenesis and which specifically incorporate labeled sulfate. It is apparent that radiosulfate uptake procedure may be used not only to study the hormonal effect on bone repair but also to investigate chondrogenesis and osteogenesis (12).

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The purpose of the present experiment was to compare the effects of cortisone and some androgenic steroids on radiosulfate uptake by the three long bones of the young cockerel's leg. An attempt was also made to offset the catabolic effect of cortisone by simultaneous treatment of birds with these anabolic androgens.

METHODS

Cockerels of White Rock-Broiler strain, used for this experiment were obtained from a commercial hatchery when three days old and immediately confined to the thermostatic brooders (Start-Gro) 24×36 inches capacity. The birds were kept in groups of fifteen per one brooder, and received a routine chicken food with tap water as desired.

The hormones were given by injection. The first dose was given on the sixth day of the bird's life and this was followed by four doses the first week and three doses weekly for the next two weeks. The following doses were used:

Cortisone (Merck) 5 mg. in 0.5 cc. of saline, subcutaneously;
17-ethyl-19-nortestosterone (Searle)*—E.N.T. 5 mg. in 0.2 cc. of sesame oil, intramuscularly;
Methyl testosterone (Ciba)—M.T. 5 mg. in 0.2 cc. of sesame oil, intramuscularly;
Testosterone propionate (Gen. Biol.)—T.P. 5 mg. in 0.2 cc. of sesame oil, intramuscularly.

Birds were divided into the following eight groups:

1. no treatment;
2. given cortisone;
3. given E.N.T.;
4. given M.T.;
5. given T.P.;
6. treated with cortisone and E.N.T.;
7. treated with cortisone and M.T.;
8. treated with cortisone and T.P.

* "Nilevar."

Isotope was administered 24 hours after the last dosage of hormone. Radiosulfate (S^{35} in H_2SO_4, Atomic Energy, Canada) was injected subcutaneously in dosage of 1 mc. per kg. of bird's body weight. The dose was dissolved in 5 ml. of distilled water together with 40 mg. of sodium sulfate (12).

Immediately after sacrifice the femur, tibiotarsus and tarso-metatarsus were separated, cleaned from adhering tissues and dried at room temperature for 24 hours, following which their weights were recorded.

The bones were then dissolved by the nitric-perchloric acid wet-digestion procedure described in detail in our previous report (13).

The final precipitate of radioactive barium sulfate was transferred to planchettes and the counting carried out using a thin mica window Tracerlab G-M tube. A Tracerlab Superscaler S.C. 18A was used for recording.

The results were calculated and presented in counts per minute (c.p.m.) per 100 mg. of dry tissue. The changes in body weight were also recorded and presented as percentages of gain in body weight, during the experimental period of three weeks.

Representative selection of bones was used for the preparation of radioautographs. For that purpose the bones were sawed in two and worn down to a flat shape. They were then placed against 1″×3″ strips of Kodak Contact Process Ortho films and exposed for 15 days at 68° F. The films were developed by routine procedure.

RESULTS

Effect of hormones on body weight. The gain of body weight observed in eight groups of cockerels during the three week experimental period is re-

TABLE 1. EFFECT OF VARIOUS STEROIDS ON BODY WEIGHT IN COCKERELS

Total dosage of each steroid was 50 mg. per bird. Results expressed as percentage of gain in body weight after three weeks of treatment. Means and S.D.

Treatment	No. of birds	% of increase in body weight	
		Mean	S.D.
Control	25	266	±10
Cortisone	18	142	± 9
E.N.T.*	18	310	±13
M.T.	15	240	± 9
T.P.	12	193	± 7
Cortisone and E.N.T.	15	228	±11
Cortisone and M.T.	15	207	± 9
Cortisone and T.P.	15	184	± 8

* E.N.T.—17-ethyl-19-nortestosterone.
M.T.—Methyl testosterone.
T.P.—Testosterone propionate.

corded in Table 1. It may be noted that normal control birds gained more than 250% of body weight as compared with about half of this value found in cortisone treated cockerels. Among the three androgens used in the present experiment, only anabolic steroid *E.N.T.* (14, 15) promotes body growth. The inhibiting effect of cortisone on body weight is, however, partially alleviated by all three androgens.

Fig. 1 shows an example of the effect of certain steroids on growth of

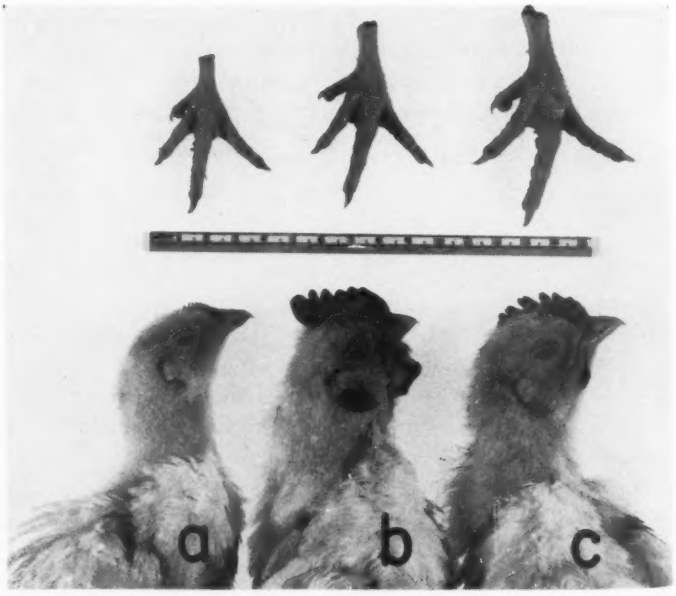

FIG. 1. Effect of cortisone (a), T.P. (b) and E.N.T. (c) on growth of chick's comb and foot.

chick's comb and foot. All three chicks were of the same body weight before treatment and weighed 155, 210 and 292 gms. respectively when sacrificed.

Inhibition of growth is well marked in the first bird (a) cortisone treated; androgenic stimulation of comb growth is best seen in the second chick (b), treated with testosterone propionate and the last one (c) demonstrates well the stimulation of growth with 17-ethyl-19-nortestosterone.

Measurement of radioactivity of bones. Table 2 records the data on S^{35} uptake by bones. It is evident that cortisone significantly reduced the radiosulfate uptake in bones as compared with normal control. The effect of this anti-anabolic hormone is more pronounced here than its influence on body growth.

Anabolic steroid 17-ethyl-19-nortestosterone (E.N.T.) significantly promoted the binding of labelled sulfate by the studied bones. Simultaneous treatment with E.N.T. and cortisone offset the effect of cortisone.

Both methyl testosterone (M.T.) and testosterone propionate (T.P.) re-

TABLE 2. RADIOSULFATE UPTAKE BY THREE LONG BONES (FEMUR, TIBIOTARSUS AND TARSOMETATARSUS) OF GROWING COCKERELS

Total dosage of each steroid per bird was 50 mg. given during 3 week period. Results expressed in c.p.m. (count per minute) per 100 mg. of bone.

Treatment	No. of birds		c.p.m. × 100
Control (no treatment)	25	Mean	6.23
		S.D.	±0.92
		Range	4.70– 7.93
Cortisone	18	Mean	1.98
		S.D.	±0.26
		Range	1.56– 2.60
E.N.T.*	18	Mean	15.17
		S.D.	±3.3
		Range	12.90–20.60
M.T.	15	Mean	8.07
		S.D.	±1.85
		Range	4.81– 9.55
T.P.	12	Mean	8.10
		S.D.	±1.76
		Range	5.45– 9.10
Cortisone and E.N.T.	15	Mean	10.18
		S.D.	±1.1
		Range	8.80–12.10
Cortisone and M.T.	15	Mean	3.51
		S.D.	±0.52
		Range	2.10– 4.63
Cortisone and T.P.	15	Mean	3.22
		S.D.	±0.48
		Range	2.00– 4.10

* E.N.T.—17-ethyl-19-nortestosterone.
M.T.—Methyl testosterone.
T.P.—Testosterone propionate.

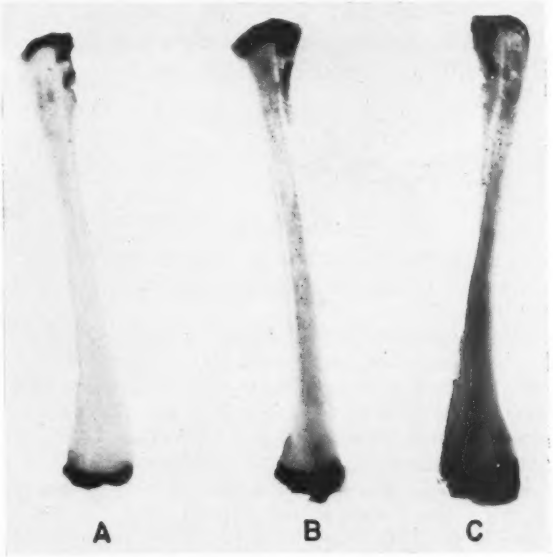

FIG. 2. Radioautographs of femora of chicks treated with cortisone (a), cortisone+E.N.T. (b) and E.N.T. alone (c).

sulted in a slight increase of S^{35} uptake by bones as compared with normal control. The effect of cortisone was partially alleviated by *M.T.* and *T.P.* These effects of *M.T.* and *T.P.* were not significant.

Fig. 2 shows the representative radioautographs of three femora. They belong to chicks treated with:

(a) cortisone;
(b) cortisone and E.N.T.;
(c) E.N.T. alone.

COMMENT

The radiosulfate uptake method confirms the previous finding (4) on inhibitory effect of cortisone on chondrogenesis and osteogenesis in birds. This isotope appears adequate for the study of hormonal effect on the production of mucopolysaccharides in connective tissue.

It is apparent that cortisone inhibits and 17-ethyl-19-nortestosterone (E.N.T.) promotes the synthesis of collagen during the process of growing of long bones in young cockerels. Combined treatment protects the connective tissue of growing bones against the anti-anabolic effect of cortisone. It may be concluded that *E.N.T.* has highly anabolic property not only during the process of healing of connective tissue (5, 6, 8) but may also accelerate the chondrogenesis and osteogenesis (12).

The androgenic steroids *M.T.* and *T.P.* which are routinely used clinically for their anabolic properties, do not show any significant effect on S^{35} uptake in the described experimental conditions.

Acknowledgment

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AUTOTRANSPLANTATION OF THE ADRENAL OF THE RAT TO THE PORTAL CIRCULATION: EFFECT OF ADMINISTRATION OF SEX HORMONES[1]

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ABSTRACT

Extensive hypertrophy occurs in an adrenal gland transplanted to the portal circulation of the adrenalectomized female rat. This hypertrophy is prevented not only by oophorectomy, but also by the administration of testosterone. Ordinarily, this phenomenon does not occur in the male rat, but can be induced in this sex by the administration of estrogen. It is inferred that estrogen is essential for, and androgen is inhibitory to, the production of this unusual hypertrophy in the transplanted adrenal.

IN A recent report, we demonstrated that an adrenal gland transplanted into the portal circulation of the adrenalectomized female rat underwent an unusual hypertrophy which was preventable by oophorectomy (1). Subsequently, it was shown that this hypertrophy did not occur in the intact or castrated male (2). The striking difference in the behavior of the sexes suggested that the production of this adrenal hypertrophy was estrogen-dependent.

The present investigation was undertaken to determine whether androgen would inhibit the production of the adrenal hypertrophy in the female and whether estrogen would induce it in the male.

METHODS

Female rats of the Long-Evans strain which weighed approximately 150 gm. were divided into five groups of fifteen animals each. In "Groups A and B," which served as intact and castrated controls respectively, only the right adrenal gland was extirpated. The remaining three groups, "C", "D", and "E" were bilaterally adrenalectomized. In "Group C" the left adrenal was re-implanted in the portal circulation (i.e., greater curvature of the stomach). "Group D" was castrated and the left adrenal similarly re-implanted in the portal circulation. The animals in "Group E" were treated the same as those in "Group C" with the additional parenteral administration of cortisone, 1 mg. daily for five days of the week. The animals in all of the groups were injected with 10 mg. of testosterone cyclopentylpropionate (Upjohn) immediately after the operation and every three weeks until sacrified.

A second similar number of male rats was prepared to correspond to "Group A, B, C, D and E" described above. However, all the male rats were injected with 1 mg. of estradiol cyclopentylpropionate (Upjohn) every 10 days postoperatively. In early

[1] This work was supported by NIH grant CY-2079 (C3) Path. and the Florence Hellman Dinkelspiel Memorial Fund for Cancer Research, Mount Zion Hospital, in the laboratories of Dr. Gerson R. Biskind.

experiments a high mortality rate was observed in animals treated with this estrogen This effect was reduced by delaying the first injection for approximately two weeks after surgery.

All of the animals were maintained on a standard laboratory diet with saline substituted for tap water during the first three weeks following surgery. The rats were weighed, sacrificed, and subjected to autopsy after 150 days. At autopsy, the adrenal gland was carefully freed of adherent tissues, blotted and weighed. If adrenal remnants were found due to incomplete removal of the gland from its original site, or if accessory adrenal tissue was discovered, the animals were not included in the series.

RESULTS

From Table 1, it is evident that after unilaterla adrenalectomy in the female rat, the administration of androgen prevents compensatory hypertrophy of the remaining adrenal left *in situ* in both the intact and castrated animal (control groups "A" and "B"). Moreover a comparison of adrenal-body weights, shows that bilateral adrenalectomy and re-implantation of one adrenal in the portal circulation of groups "C" and "D" treated with androgen, does not result in significant adrenal hypertrophy in either the intact or castrated animal. This is in sharp contrast to what was reported in females not receiving testosterone (1). Although groups "C" and "D" do show a significant increase in absolute adrenal weight when compared with "A" and "B," the largest absolute adrenal weight of 42.5 mg. found in group "D" treated with testosterone, is less than the 52.5 mg. absolute weight previously reported for the untreated unilateral adrenalectomized control (1).

TABLE 1. EFFECT OF TESTOSTERONE ON MEAN CHANGE IN ADRENAL WEIGHT AFTER 150 DAYS IN FEMALE RATS

Type of experiment	No. of rats	Initial wt.* (Rt. adrenal)	Final wt.* (Lt. adrenal)	Change in weight	S.D. of change in weight
A. Left adrenal in situ	14	13.8 (22.3)†	8.4 (21.5)†	−5.4	2.5
B. Left adrenal in situ with castration	15	13.5 (22.9)	9.0 (22.1)	−4.5	3.0
C. Left adrenal in portal circulation	12	11.8 (18.0)	15.4 (35.3)	3.6	5.9
D. Left adrenal in portal circulation with castration	12	12.5 (19.7)	16.7 (42.5)	4.2	9.0
E. Left adrenal in portal circulation castration, parenteral cortisone 5 mg./week	12	12.8 (19.1)	14.2 (36.6)	1.4	6.5

* The weight of the adrenal is expressed as the mean weight in mg. per 100 gm. body weight. Testosterone was administered as the cyclopentylpropionate, 10 mg. every 3 weeks postoperatively.

† All numbers in parentheses are the mean actual weights of the adrenals in mg.

TABLE 2. EFFECT OF ESTROGEN ON MEAN CHANGE IN ADRENAL WEIGHT AFTER 150 DAYS IN MALE RATS

Type of experiment	No. of rats	Initial wt.* (Rt. adrenal)	Final wt.* (Lt. adrenal)	Increment	S.D. of Increment
A. Left adrenal in situ	15	7.2 (18.3)‡	14.3 (33.0)	7.1	2.6
B. Left adrenal in situ with castration	15	7.4 (17.5)	13.4 (35.0)	6.0	1.9
C. Left adrenal in portal circulation	9	6.1 (13.1)	31.7 (83.4)	25.6	8.6
D. Left adrenal in portal circulation with castration	10	6.6 (15.7)	40.7 (102.7)	34.1	10.5
E. Left adrenal in portal circulation, castration, parenteral cortisone, 5 mg./week†	9	7.0 (17.1)	30.1 (65.1)	23.1	11.6

* The weight of the adrenal is expressed as the mean weight in mg. per 100 gm. body weight. Estrogen was administered as estradiol cyclopentylpropionate, 1 mg. every 10 days post-operatively.

† Average age at autopsy was 126 days after operation due to the poor condition of the animals.

‡ All numbers in parentheses are the mean actual weights of the adrenals in mg.

From Table 2, it is evident that the administration of estrogen to the male rat results in some compensatory hypertrophy of the remaining adrenal after unilateral adrenalectomy (control groups "A" and "B"), and marked hypertrophy in the intraportal adrenal of both the intact and castrated animal (groups "C" and "D"). This, again, is in sharp contrast to the findings reported in the absence of administered estrogen where no adrenal hypertrophy occurred in either the intact or castrated male rat under any of the experimental conditions.

The effect of cortisone in the manner and dosage administered in these experiments in negligible.

The histological appearance of the adrenals and pituitary glands was similar to that reported in a previous communication (1) with the additional expected known changes observed in the pituitary due to the administration of androgen or estrogen.

In many instances deposition of calcium occurred in the transplanted adrenals. Since similar deposits of calcium occurred in the transplanted gland whether hypertrophied or not, this finding does not alter the interpretation of the changes in adrenal weight reported in these experiments.

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SOME EXTRA-ADRENAL ACTIONS OF CORTICOTROPIN ON CARBOHYDRATE METABOLISM IN THE RAT[1]

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ABSTRACT

ACTH induces a fall in blood sugar one and two hours after its injection into acutely adrenalectomized fasted rats maintained with hydrocortisone hemisuccinate. The hypoglycemic effect is abolished when the hormone is inactivated by boiling in 0.1N NaOH or oxidation with H_2O_2. In the latter case hypoglycemic activity is restored by reduction with cysteine, just as occurs with its adrenal-stimulating activity. In non-fasted intact and adrenalectomized rats given 2 ml. of 50% glucose by stomach tube ACTH brings about a significant lowering of the glucose tolerance curve and an increase in dorsal brown adipose tissue glycogen in 3 hours, with no change in liver glycogen. In these respects it simulates the action of insulin and growth hormone. Hydrocortisone hemisuccinate has no influence on the glucose tolerance or adipose tissue glycogen, but significantly increases liver glycogen. The adipose tissue glycogen response is abolished when ACTH is inactivated by NaOH or H_2O_2 and is restored when the latter is reactivated with cysteine. Like growth hormone, ACTH has no influence on adipose tissue glycogen in acutely eviscerated (depancreatized) rats given glucose, whereas insulin is as effective as in intact rats. ACTH does not potentiate the effect of a small dose of insulin. From these results, it is concluded that ACTH, like growth hormone, stimulates the secretion of insulin by an extra-adrenal mechanism, but evidence is presented which indicates that this is not the sole mechanism by which ACTH induces hypoglycemia and improved glucose tolerance in the rat. ACTH also modifies glucose tolerance in the eviscerated rat, suggesting an extra-pancreatic action, but reasons are given for accepting this interpretation cautiously. These data are presented as additional evidence that ACTH may influence metabolism independently of the adrenal cortex and that its actions are not accounted for by contamination with growth hormone or other pituitary factors.

IN 1954 Westermeyer and Raben reported that an anterior pituitary extract prepared by the glacial acetic acid-oxycellulose method and rich in corticotropin and intermedin was active in lowering the blood sugar in intact and adrenalectomized mice (1). Independently, similar observa-

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tions were made in this laboratory (2). In general, the effects on glycemia were similar to those which had been originally described for a crude thyrotropin preparation by Horsters (3), for what were considered thyrotropin-free pituitary extracts by Hoffmann and Anselmino (4) and by O'Donovan and Collip (5), and for purified growth hormone by Milman and Russell (6).

The present study presents additional data to support the view that ACTH does indeed have extra-adrenal effects on carbohydrate metabolism which are intrinsic properties of ACTH rather than being due to contamination with other hormones. This action is only one of several metabolic actions of ACTH which have been described and interpreted by us as representing extra-adrenal actions of this hormone (7). Evidence will be presented suggesting that *one* extra-adrenal mechanism by which ACTH influences carbohydrate metabolism is by stimulation of insulin secretion by the pancreas.

MATERIALS AND METHODS

Male albino rats of the Vanderbilt strain, weighing 150–250 grams were used. Nembutal anesthesia was employed for adrenalectomy, functional evisceration (8) and when rats were sacrified for tissue analyses. Otherwise, all studies were carried out in unanesthetized rats. Blood samples were obtained from the tail, the rats being lightly restrained by wrapping in towels. Blood was analyzed for glucose by the method of Somogyi (9) and the liver and dorsal interscapular adipose tissue for glycogen by the anthrone method (10).

The ACTH preparations used included two lots of oxycellulose adsorbed ACTH especially prepared for us by the Wilson Laboratories (Lot #102621, 140 U.S.P. units/mg. and Lot #102201, 90 U. S. P. units/mg.), and α_1-α_2 ACTH from the American Cyanamid Company. The latter was a mixture of ACTH peptides derived by purification of oxycel ACTH by countercurrent distribution and assaying 80–100 U. S. P. units/mg. (11). In addition an old sample of Armour ACTH, presumably protein in nature (Lot J17409R, 1 unit/mg.) was available.

RESULTS

The blood glucose lowering effect of ACTH was tested in rats which were adrenalectomized at the 24th hour of fasting and given 1 mg. of hydrocortisone hemisuccinate intraperitoneally at the time of operation. Three to four hours postoperatively blood was removed from the tail for glucose determination and the test hormone injected intraperitoneally. Subsequent blood samples were taken at 1 and 2 hours. The results are recorded in Table 1. Control animals receiving 1 mg. of human serum albumin showed no significant change in blood sugar whereas those in which 1 mg. of oxycel ACTH was injected intraperitoneally exhibited falls of 24 ± 3.1 and 26 ± 2.7 mg.% at 1 and 2 hours respectively. ACTH is known to resist mild treatment with alkali (24 hours in 0.1N NaOH at 25° C) but its adrenal ascorbic acid depleting activity is lost on boiling the hormone in 0.1N NaOH for 20 minutes (12). Furthermore, it has been

found that ACTH may be reversibly inactivated by treatment with hydrogen peroxide, activity being restored by incubation with cysteine (13). The results of these procedures on the hypoglycemic effect of ACTH are seen in Table 1. The mild alkali treatment did not modify the hypoglycemic response, whereas boiling in alkali abolished it, as did H_2O_2 treatment. The H_2O_2 treated hormone, however, could be reactivated with cysteine. These responses to inactivation procedures thus correspond exactly with the known effects of these procedures on adrenal ascorbic acid depleting activity of ACTH. Protein ACTH induced some hypoglycemia but it was not as prompt or striking as with the oxycel and α_1–α_2 ACTH.

TABLE 1. EFFECT OF ACTH ON BLOOD GLUCOSE IN ACUTELY ADRENALECTOMIZED FASTED RATS RECEIVING 1 MG. HYDROCORTISONE HEMISUCCINATE INTRAPERITONEALLY

Hormone	Treatment	No. of rats	Blood glucose, mg./100 ml. ±S.E.		
			Initial	Change in	
				1 hour	2 hours
Control—Albumin	—	13	66 ± 2.4	− 2 ± 3.0	− 6 ± 3.9
1 mg. Oxycel ACTH	—	8	75 ± 2.0	−29 ± 3.1**	−26 ± 2.7**
1 mg. Oxycel ACTH	NaOH, 24′, 25°	6	75 ± 5.4	−30 ± 7.1**	−24 ± 7.1**
1 mg. Oxycel ACTH	NaOH, 20″, 100°	6	72 ± 6.0	− 2 ± 1.6	− 2 ± 1.8
1 mg. Oxycel ACTH	H_2O_2	9	66 ± 2.2	+ 5 ± 3.5	− 9 ± 3.6
1 mg. Oxycel ACTH	H_2O_2 + Cysteine	8	75 ± 4.4	−34 ± 3.7**	−27 ± 4.2**
.10 mg. Protein ACTH	—	12	65 ± 1.9	−10 ± 3.9†	−19 ± 3.4*

** $P < .01$ compared to control.
* $P < .02$ compared to control.
† 6 observations.

In a previous report it was shown that although growth hormone had no detectable influence on glucose tolerance in fed rats it did induce a significant accumulation of glycogen in the dorsal interscapular fat pad of the rat following a glucose load (14). Since STH did not exhibit the latter effect in the rat acutely depancreatized by evisceration, whereas insulin did in both the intact and eviscerated rat, it was concluded that STH stimulates insulin secretion in the fed rat. In view of the many similarities in metabolic response to STH and the extra-adrenal actions of ACTH (7) observations were made on glucose tolerance and liver and adipose tissue glycogen levels of fed rats given ACTH.

Table 2 records the data from an experiment in which intact non-fasted rats weighing between 150 and 200 grams were tube-fed 2 ml. of 50% glucose and their blood was sampled for glucose determination prior to and 1, 2, and 3 hours after intubation. At 3 hours they were sacrificed under nembutal anesthesia and samples of dorsal interscapular adipose tissue and liver removed for glycogen determination. The controls received only glucose and the insulin group was injected subcutaneously with 0.5 unit of

glucagon-free insulin at the same time as the glucose intubation. These first two groups also served as the controls for the previous study (14) which was conducted simultaneously with the present one. The four experimental groups received either 10 mg. of protein ACTH, 2 mg. of α_1–α_2 ACTH, 2 mg. of oxycel ACTH or 10 mg. of hydrocortisone hemisuccinate respectively.

Insulin, as anticipated, caused a significant lowering of the glucose tolerance curve and an elevation in adipose tissue glycogen, but there was no change in liver glycogen. Both oxycel and α_1–α_2 ACTH likewise significantly improved glucose tolerance, but to a lesser degree than insulin. On the other hand, both preparations of ACTH brought about increases in adipose tissue glycogen which were quite comparable to those from 0.5 U.

TABLE 2. GLUCOSE TOLERANCE AND LIVER AND ADIPOSE GLYCOGEN LEVELS IN NON-FASTED, INTACT RATS GIVEN 2 ML. OF 50% GLUCOSE BY STOMACH TUBE

Experimental group	Blood glucose, mg. per 100 ml.[1]				Glycogen, gm. per 100 gms.[1]	
	Initial	Change in				
		1 hour	2 hours	3 hours	Adipose tissue	Liver
Control (15)	115±2.0	+28±3.4	+33±2.9	+19±2.9	0.277±0.054	4.42±0.312
0.5 U. Insulin (10)	120±4.0	−35±4.1**	−30±4.0**	− 2±7.2**	0.820±0.127**	4.00±0.170
10 mg. Protein ACTH (17)	113±1.6	+18±1.9*	+23±2.4*	+17±3.0	0.389±0.046	4.61±0.420
2 mg. α_1-α_2 ACTH (11)	118±2.4	+ 6±4.7**	+21±3.6*	+17±3.8	0.727±0.104**	4.77±0.352
2 mg. Oxycel ACTH (12)	113±2.5	+ 3±2.5**	+26±3.9	+26±2.5	0.812±0.101**	5.05±0.289
10 mg. Hydrocortisone Hemisuccinate (12)	111±1.9	+32±3.2	+32±3.0	+21±1.4	0.149±0.029	6.99±0.080**

[1] Mean ± S.E.
** P < .01.
* P < .02.

of insulin. ACTH did not modify the liver glycogen. Protein ACTH produced similar but lesser effects in glucose tolerance but did not significantly modify adipose tissue or liver glycogen. The hydrocortisone hemisuccinate had no detectable influence on either glucose tolerance or adipose tissue glycogen, but did significantly increase liver glycogen. In these respects it differed notably from ACTH suggesting that the latter, which simulated the growth hormone in its action (14), did not act by stimulation of the adrenal cortices. However, this conclusion cannot be accepted with confidence since corticosterone and not hydrocortisone is the major secretory product of the adrenal cortex in the rat.

For this reason the experiment with oxycel ACTH was repeated using rats which had been adrenalectomized and given 1 mg. of hydrocortisone hemisuccinate intraperitoneally 4 hours prior to beginning the experiment. The results, plotted in Figure 1, reveal the effects of ACTH even more dramatically than in the intact animal. Adipose tissue glycogen was again significantly increased after ACTH, with no change in liver glycogen, and an impressive improvement in glucose tolerance occurred. The change in glucose tolerance demonstrated here in both the intact and adrenalec-

tomized rats confirms results previously published (15). These data clearly establish the extra-adrenal nature of the ACTH effect on glucose tolerance and adipose tissue glycogen.

Further evidence for the specificity of the ACTH effect on the adipose tissue glycogen levels is found in the inactivation studies in Table 3. As noted with the hypoglycemia response to ACTH in Table 1 the adipose tissue glycogen accumulation did not occur when ACTH was inactivated by boiling in alkali or oxidation with H_2O_2. In the latter case incubation of the H_2O_2 inactivated hormone with cysteine resulted in full restoration of activity.

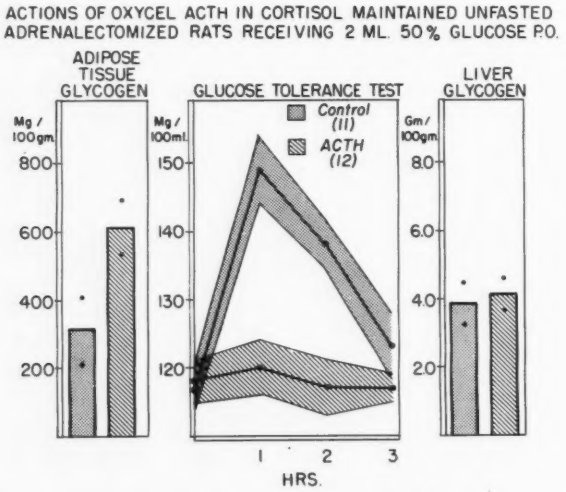

FIG. 1. Adipose tissue glycogen, glucose tolerance, and liver glycogen (mean ± S.E.) in cortisol-maintained adrenalectomized, non-fasted rats, receiving 2 ml. of 50 per cent glucose by stomach tube and 2 mg. of oxycel ACTH intraperitoneally.

The previous study with growth hormone showed that the adipose tissue glycogen response to STH was lost when the animals were depancreatized by functional evisceration (14). Identical observations were made simultaneously with ACTH and the data are recorded in Table 4 using the same controls and insulin values as in the previous report. The animals, which were not fasted, were eviscerated under nembutal anesthesia just prior to receiving ACTH by slow intravenous injection and 1 ml. of 20% glucose subcutaneously in each of 4 separate sites (800 mg.). Insulin was also injected subcutaneously, but well removed from the sites of glucose injection. Between blood sampling the animals were kept in a chick-incubator at 33° C to maintain body temperature. The controls showed a markedly diabetic glucose tolerance curve, as expected in the depancreatized, liver-

TABLE 3. EFFECT OF INACTIVATION PROCEDURES ON ACTH EFFECT ON ADIPOSE TISSUE GLYCOGEN IN NON-FASTED RATS

Hormone-Treatment	No. of rats	Adipose tissue glycogen, gm./100 gm., Mean ± S.E.
Control	15	0.277 ± 0.054
2 mg. Oxycel ACTH	16	0.606 ± 0.113**
2 mg. Oxycel ACTH, NaOH, 20", 100°	16	0.310 ± 0.056
2 mg. Oxycel ACTH, H_2O_2	8	0.242 ± 0.063
2 mg. Oxycel ACTH, H_2O_2 + Cysteine	8	0.586 ± 0.137*

** P < .01.
* P < .05.

less rat, and there was no significant accumulation of glycogen in the adipose tissue. This suggests that the glycogen levels achieved in the intact animals receiving glucose alone are dependent on endogenous insulin secretion. When the eviscerated rats were given 0.05 unit of insulin, glucose tolerance was significantly improved, but not restored to normal, and a definite increase in adipose tissue glycogen occurred. With 0.5 unit of insulin, blood sugar levels were restored more nearly to normal and the adipose tissue glycogen level was indistinguishable from that of the intact rat given the same dose of insulin and approximately the same glucose load (Table 2). Two mg. of either oxycel or α_1–α_2 ACTH were totally without effect on the adipose tissue glycogen. The glucose tolerance curve was significantly lower in the oxycel ACTH group than in the controls. However, it was noted that these animals reacted less favorably to the intravenous ACTH injection, frequently becoming cyanotic and showing irregular respiration for a time. Since peripheral circulatory failure in the eviscerated rat is associated with an accelerated fall in blood sugar (16) we feel that the lower glucose curve should be interpreted cautiously. When the more highly purified α_1–α_2 ACTH was used the above circulatory and respiratory changes were not apparent and the difference in the glucose tolerance curve was abolished except for the 3rd hour at which time the blood sugar was still significantly lower in the ACTH group. The possible significance of this is discussed below. In the last group 0.05 unit of insulin and 2 mg. of

TABLE 4. GLUCOSE TOLERANCE AND ADIPOSE TISSUE GLYCOGEN IN NON-FASTED EVISCERATED RATS GIVEN 8 ML. 10% GLUCOSE S.Q.

Experimental group	Blood glucose, mg. per 100 ml.[1]				Adipose tissue glycogen, gm./100 gm.[1]
	0	1 hour	2 hours	3 hours	
Control (6)	120 ± 7.5	477 ± 33.8	548 ± 38.9	602 ± 26.8	0.051 ± 0.012
0.05 u. Insulin (7)	112 ± 2.3	409 ± 9.2	430 ± 34.3†	430 ± 41.6**	0.111 ± 0.023†
0.5 u. Insulin (5)	99 ± 5.2	231 ± 29.6**	150 ± 13.5**	108 ± 43.9**	0.916 ± 0.156**
2 mg. α_1-α_2 ACTH (6)	105 ± 19.1	432 ± 25.7	511 ± 28.9	475 ± 24.6**	0.019 ± 0.004
2 mg. Oxycel ACTH (6)	104 ± 18.5	370 ± 13.8*	439 ± 22.0†	427 ± 30.7**	0.034 ± 0.006
2 mg. Oxycel ACTH + 0.05 u. Insulin (6)	102 ± 2.6	388 ± 29.4	440 ± 49.0†	467 ± 149.0**	0.047 ± 0.008

[1] Mean ± S.E.
** P < .01.
* P < .02.
† P < .05.

oxycel ACTH were given simultaneously in order to determine whether the ACTH effect on adipose tissue depends simply on insulin being available or whether functional islet tissue is required. It was found that ACTH did not potentiate the insulin response and hence the actions of ACTH in increasing adipose tissue glycogen in the intact animal cannot be explained by an enhancement of insulin action.

DISCUSSION

These experiments present further evidence that certain corticotropin preparations are capable of influencing metabolism independently of their effect on the adrenal cortex and that their action is due to the corticotropin itself and not to its contamination with other known hormones. The alterations in carbohydrate metabolism are readily demonstrable in adrenalectomized animals and hence adrenal mediation is clearly ruled out. They appear to be specific in the sense that procedures known to inactivate and reactivate the adrenal ascorbic depleting potency of ACTH influence the extra-adrenal carbohydrate metabolic activities in an identical manner. The melanophore stimulating hormone is also reversibly inactivated and reactivated by H_2O_2 and cysteine (17) but differs from ACTH in that it is not destroyed by boiling in alkali (18) and hence it is probably not responsible for the observed effects. Although growth hormone has been shown to have similar effects on carbohydrate metabolism in the rat it seems unlikely that the ACTH responses noted here are due to contamination with growth hormone since larger doses of the latter are required to induce comparable effects (14). Furthermore, preliminary data indicate that growth hormone is not inactiviated by H_2O_2. Finally, vasopressin, which was found by Ingle and Li (19) to be responsible for the "extra-adrenal" action of ACTH in the muscle-work test, would not be expected to be reversibly inactivated by H_2O_2 and cysteine.

An old sample of protein ACTH was partially active, contrasting with its inactivity in inducing ketosis previously reported (12). Since it has now been found that as little as 10 μg. of the same ACTH preparation will stimulate lipolysis in adipose tissue in vitro whereas 200–1000 μg. of different samples of STH are required to bring about the same effect (20) it may be safely concluded that this ACTH effect cannot be attributed to growth hormone contamination. These recent demonstrations of extra-adrenal effects with protein ACTH make it necessary to abandon the suggestion previously made that the extra-adrenal effects of ACTH might be peculiar to the peptide preparations (7, 12).

The metabolic responses to ACTH in both intact and adrenalectomized rats simulated those to growth hormone, but in general were more intense. In our experience, hypoglycemia is not regularly induced by all samples of growth hormone, even in adrenalectomized rats, whereas the ACTH has been uniformly effective. ACTH improved glucose tolerance in both

intact and adrenalectomized rats, whereas growth hormone had no comparable effect in our hands (14) although Maassen has described such a response (21). Both hormones impressively increased adipose tissue glycogen in the intact but not in the eviscerated rat, but twice as much growth hormone was required to produce the same response as ACTH. Neither hormone changed liver glycogen levels under the conditions of these experiments.

We have previously presented the justification for interpreting the adipose tissue glycogen response as an indication of insulin secretion (14). In brief, it was noted that insulin stimulated glycogen accumulation irrespective of whether the rats were fasted, fed, intact or eviscerated, whereas a response to growth hormone as well as to such agents as tolbutamide (22) took place only when the pancreas was not only intact, but presumably contained adequate stores of insulin. Thus, growth hormone and tolbutamide brought about adipose tissue glycogen deposition only in the intact fed rat, but not in the fasted or eviscerated animal. The former has low insulin stores while the latter lacks the pancreas. The response to ACTH in this test was essentially the same as that to growth hormone and hence may be similarly interpreted as indicating that this hormone stimulates insulin secretion. This response takes place independently of the adrenal cortex, judging from the results in the adrenalectomized animals and the lack of response to hydrocortisone administered acutely.

It is not justified to conclude from these data, however, that insulin secretion accounts entirely for the blood sugar response to ACTH and STH. Indeed, the hypoglycemia actually occurs in the face of other reactions which may be interpreted as anti-insulin. Westermeyer and Raben (1) reported insulin resistance in adrenalectomized mice receiving oxycel ACTH. ACTH induces ketosis as rapidly as hypoglycemia (2) in contrast to insulin and tolbutamide which first lower blood ketones and lead to ketosis only after several hours (22). One way to reconcile these discrepancies is by assuming that ACTH may block certain actions of insulin, such as on the liver, whereas it does not prevent the insulin action on brown adipose tissue which is known to be extremely sensitive to this hormone (23). At the same time, ACTH may have other actions on carbohydrate metabolism which are either entirely independent of insulin or are synergistic with it. Astwood has reported effects of ACTH in increasing cardiac glycogen (24) whereas Russell has shown that insulin does not have this property (25). Randle has shown that ACTH, like growth hormone, enhances the action of insulin on the uptake of glucose by the isolated diaphragm (26). Our data do not reveal a similar interaction between insulin and ACTH with respect to glucose tolerance or adipose tissue glycogen in the eviscerated rat. The improved glucose tolerance in the eviscerated rat which received α_1–α_2 ACTH and did not exhibit visible circulatory distress may indicate a direct action of ACTH on peripheral carbohy-

drate utilization. It is obvious that considerable more investigation is necessary before the mechanism(s) of the extra-adrenal actions of ACTH in carbohydrate metabolism will be fully understood.

It should be emphasized that very large doses of ACTH were employed in this study. The experiments were not designed to determine whether ACTH as physiologically secreted exerts extra-adrenal metabolic actions. The present study only shows that ACTH is capable of influencing carhydrate metabolism in a manner which is virtually indistinguishable from that of growth hormone. At the moment the significance of this observation has bearing chiefly on our concepts of the mechanism of action and specificity of pituitary hormones (7). Only the future will tell whether the alterations noted on injection of very large doses of ACTH have any counterpart in actions of endogenously secreted ACTH in either the intact or adrenalectomized animal. And finally, although a variety of techniques were employed in an attempt to identify the biological reactions with ACTH itself, it must be recognized that the final proof of identity must await repetition of these experiments with samples of ACTH of unassailable purity. Because of limitations of supply it has been possible to test the most pure corticotropin available, Corticotropin A, for only two extra-adrenal actions so far, ketogenic (12) and lipolytic (20). In both cases it was as active as the preparations of ACTH used in the present study. This contrasts with Ingle and Li's experience in which the most highly purified ACTH was distinctly less active in the muscle-work test than either cruder ACTH preparations or vasopressin (19).

Acknowledgments

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Note added in proof:

Although no synergism or antagonism between insulin and ACTH on glycogen accumulation in brown adipose tissue could be demonstrated in the present studies, preliminary observations indicate that ACTH strongly inhibits the action of insulin in promoting glucose uptake by white adipose tissue in vitro. (J. Verner and F. L. Engel, to be published.)

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THE QUANTITATIVE HISTOLOGICAL DISTRIBUTION OF THE SUCCINIC DEHYDROGENASE SYSTEM IN THE RAT ADRENAL AND THE INFLUENCE OF HYPOPHYSECTOMY, ACTH, AND CORTISONE[1,2]

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ABSTRACT

No significant differences were detected in the quantitative histological distribution of the succinic dehydrogenase system in adrenals from adult male albino rats sacrificed at midnight and at noon. Peak activities were found in the reticularis. Subcutaneous injections of 25 mg. ACTH per kg. 3 hours before sacrifice had little if any effect on the enzyme activity per unit protein-nitrogen in the various histological zones of the rat adrenal. However, daily subcutaneous injections of 7 mg. cortisone acetate per kg. for 2 weeks before sacrifice elicited some increase in all zones. Six days after hypophysectomy lowered activity was found only in the reticular and in the mixed reticular and medullary zones.

THE term "succinic dehydrogenase system" is employed as suggested by Farber *et al.* (1) and Singer and Kearney (2) for the coupled reactions involving enzymatic oxidation of succinate in the presence of intermediate factors and electron acceptors such as tetrazolium salts. The initial study in this laboratory on the succinic dehydrogenase system in the adrenal gland dealt with the development of a colorimetric method, based on the use of neotetrazolium chloride (NT) as the hydrogen acceptor, for measurement of the enzyme system in microgram samples of tissue, and the application of this method to the histological distribution of the enzyme system in the rat adrenal (3). Subsequently, a technically simpler procedure was employed in which 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INPT) was used as the hydrogen acceptor to produce a formazan stain which could be extracted and measured colorimetrically. Comparison of both procedures in a study of the histological distribution of the enzyme system in the steer adrenal revealed that they were in agreement, although the latter was more sensitive (4). The method using INPT

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proved to be preferable for microtome sections of tissues, but because of the short digestion period of 5 minutes, it was not as practical as the NT method for analysis of tissue homogenates.

The present investigation was designed to determine what influence might be exerted on the concentration of the succinic dehydrogenase system in the separate zones of the rat adrenal gland by sacrifice of untreated animals at noon and at midnight, by hypophysectomy and administration of ACTH and of cortisone.

EXPERIMENTAL

Male albino rats (Sprague-Dawley) weighing 350–500 gm. were used in all of the experiments. For the hypophysectomy experiments unoperated, sham-operated and hypophysectomized rats were obtained from the Hormone Assay Laboratories, Inc., Chicago. For the other work the rats were obtained from the Holtzman Co., Madison, Wis., and since groups of these animals were obtained in different seasons of the year for separate experiments, appropriate controls from each group were used.

The animals were housed in a constant climate room at 25° ± 1° C, 30–50% relative humidity, and controlled electrical illumination, 12 hours of light (6 A.M. to 6 P.M.) and dark. Instantaneous death was inflicted by a single hammer blow on the head. The adrenals were quickly removed, frozen with solid carbon dioxide and stored at −20° C. The procedures for obtaining the microtome sections of fresh frozen adrenal tissue for chemical analysis and histological examination were modifications of those previously employed (5,6). Newer equipment for obtaining and sectioning tissue samples was employed (7), and the method of placing the sections to be used for enzyme measurement on cover glasses, flattening them, and thawing for a few moments on an electrical heater to make them adhere firmly to the glass, followed the earlier description (4).

For each tissue section used for a separate chemical analysis, the previous adjacent section was taken for histological identification after staining with toluidine blue, and the following adjacent section was used for protein-nitrogen determination. The section to be stained for identification was brushed flat on an albuminized slide and dried in a desiccator to make it adhere firmly to the slide. Then it was passed through 70% and 30% alcohol, 30 seconds each, before staining with 0.5% toluidene blue in 20% alcohol containing 1% acetic acid.

Determination of the activity of the succinic dehydrogenase system in the tissue sections followed the procedure already given (4) with the difference that 50 μl. of tetrachloroethylene was used to extract the formazan from the dried section. Since this volume was large enough to permit use of Lowry-Bessey cuvettes (8) in the Beckman spectrophotometer, this equipment was employed and readings were taken at 505 mμ.

Protein-nitrogen in tissue sections was measured by the bromsulfalein procedure (9).

RESULTS

It is apparent from Figures 1 and 2 that no significant difference in either the distribution or magnitude of the enzyme system in the rat adrenal was detectable between the groups of animals sacrificed at midnight and noon. The lower activities observed in the adrenals of the untreated group (Fig. 3), sacrificed at 11:30 A.M., probably were due to the fact that this was a different group of animals obtained over a year earlier from different stock.

A single subcutaneous injection of 25 mg. ACTH per kg. 3 hrs. before sacrifice produced some elevation of the enzyme activity in all zones of the adrenal when the activity was based on volume of the fresh tissue (Fig. 3), but this effect was practically abolished when the activity was referred to the protein-nitrogen content. When rats in this group were given daily

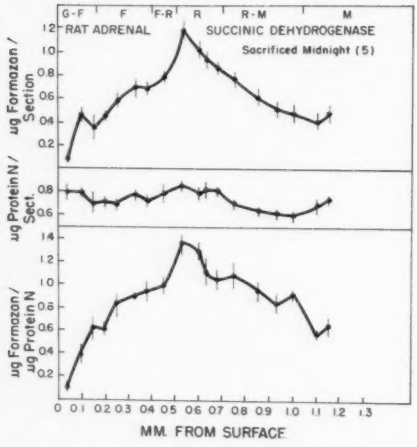

FIG. 1. Distribution of the INPT activity of the succinic dehydrogenase system and protein-nitrogen in the right adrenal glands of adult male albino rats sacrificed at midnight. Sections 1.5 mm. in diameter, 16 μ thick (0.028 μl. vol.). In this, and in the subsequent figures, the following apply: in curves of activity per tissue section and of protein-nitrogen per section, the points represent means and the vertical lines through them the standard error of the means. In curves of activity per unit protein-nitrogen, the points and vertical lines are ratios of the corresponding values for activities per section

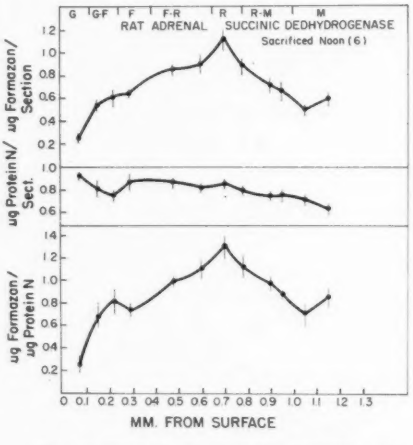
G G-F F F-R R R-M M
RAT ADRENAL
SUCCINIC DEHYDROGENASE
Sacrificed Noon (6)
µg Formazan / Section
µg Protein N / Sect.
µg Formazan / µg Protein N
MM. FROM SURFACE

FIG. 2. Distribution of the INPT activity of the succinic dehydrogenase system and protein-nitrogen in the right adrenal glands of adult male albino rats sacrificed at noon. Sections 1.5 mm. in diameter, 16 μ thick (0.028 μl. vol.).

to protein-nitrogen per section. Histological zones are indicated by C (capsule), G (glomerulosa), F (fasciculata), R (reticularis), M (medulla). When sections are mixtures of two zones both letters are used. Numbers in parenthesis denote the number of adrenals analyzed to obtain the composite curve.

subcutaneous injections of 7 mg. cortisone acetate per kg. for 2 weeks prior to sacrifice, a small rise in activity was observed on the basis of fresh volume, with a little greater elevation when referred to protein-nitrogen.

After hypophysectomy lowered activity was found 6 days after operation only in the reticular and in the mixed reticular and medullary zones (Fig. 4). The reduction in size following the atrophy induced by hypophysectomy is apparent from the abscissae; the decrease in weight per gland was 50–60%.

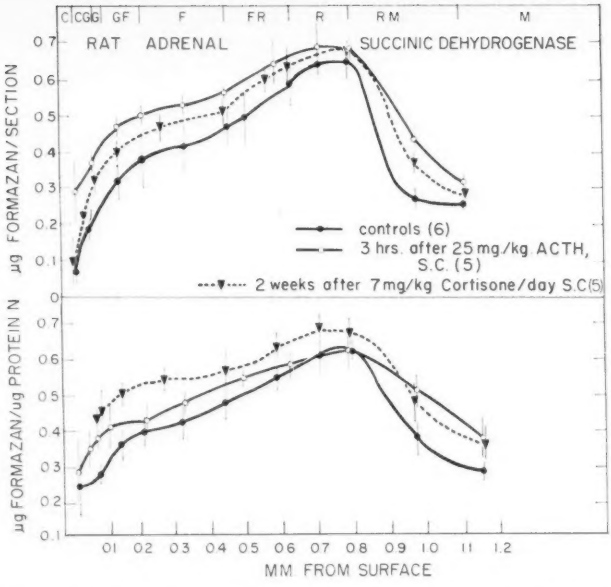
C C G G GF F FR R RM M
RAT ADRENAL SUCCINIC DEHYDROGENASE
µg FORMAZAN/SECTION
controls (6)
3 hrs. after 25 mg./kg. ACTH, S.C. (5)
2 weeks after 7 mg/kg. Cortisone/day S.C.(5)
µg FORMAZAN/µg PROTEIN N
MM. FROM SURFACE

FIG. 3. Effect of single subcutaneous injections of ACTH, and of daily subcutaneous injections of cortisone for 2 weeks, on INPT activities of the succinic dehydrogenase system in adrenal glands of adult male albino rats. Sections 1.5 mm. in diameter, 16 μ thick (0.028 μl. vol.).

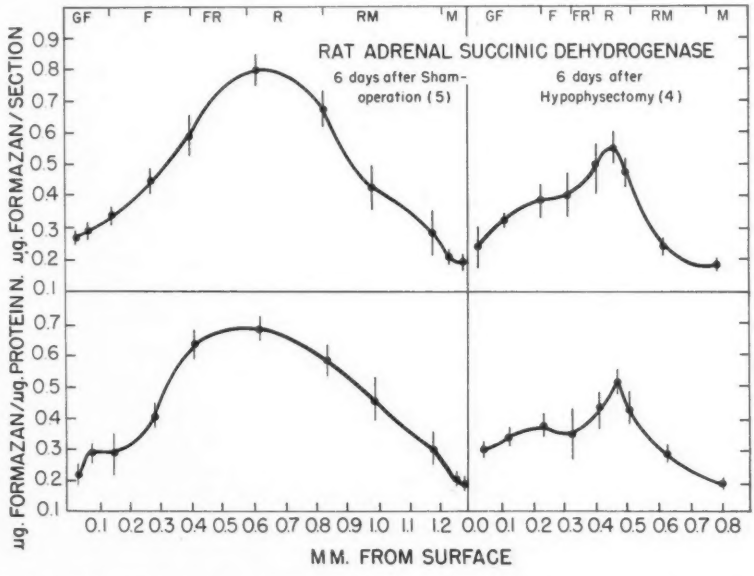
GF F FR R RM M GF F FR R RM M
RAT ADRENAL SUCCINIC DEHYDROGENASE
6 days after Sham-operation (5)
6 days after Hypophysectomy (4)
µg. FORMAZAN/SECTION
µg. FORMAZAN/µg. PROTEIN N.
MM. FROM SURFACE

FIG. 4. Effect of hypophysectomy on INPT activities of the succinic dehydrogenase system in right adrenal glands of adult male albino rats. Sections 1 mm. in diameter, 16 μ thick (0.013 μl vol.).

DISCUSSION

Wet weight of tissue is usually considered a poorer basis than dry weight to which to refer enzyme activity, because concentration data might be affected by changes in tissue hydration. With adrenal tissue, even dry weight is an unfavorable basis of reference because of the unusually high concentration of lipides which may constitute over half of the total dry material in the fascicular and reticular regions. For this reason activity has been expressed in terms of protein-nitrogen which represents the essential protoplasm of tissue.

It is apparent from Fig. 3 that even intense stimulation of the gland with ACTH had little effect on the activity per unit protein-nitrogen in the various adrenal zones, although a tendency to elevated values was found when the activity was referred to volume of fresh tissue. Furthermore, the lack of influence on either the histological distribution or magnitude of the enzyme activity observed (Figs. 1 and 2) when the animals were sacrificed at midnight or noon, the times of their most and least intense bodily activity, also appears to be consistent with these findings. This is of particular interest because the well established work of Halberg and coworkers has implicated the adrenal in the control of diurnally varied functions (10). In work to be reported subsequently, Greenberg and Glick found that coenzyme A and labile phosphate esters in the rat adrenal do undergo noon-midnight changes. It should be emphasized that the lack of a midnight-noon difference in no way eliminates the possibility of diurnal changes, which still might exist within the 12 hour period employed.

The lack of an ACTH effect is in agreement with the results of Kuusisto and Telkka (11) who found no influence on the enzyme system visualized by NT staining of the rat adrenal after the adult animals received daily intramuscular injections of 15 mg. of ACTH per kg. for 5 days. This stands in contrast to the earlier report of Zweifach *et al.* (12) that 20 μg. of ACTH injected intramuscularly twice daily for 3 days into rats caused decreased staining by triphenyltetrazolium chloride (TPT) in the fascicular and reticular zones of the adrenal. This result may have followed the loss in lipide induced in these cortical regions by ACTH, since it is known that TPT formazan is somewhat soluble in lipide, as Perry and Cumming (13) pointed out. NT formazan is also lipide soluble but it is formed more quickly and is more easily detected in sections than the TPT formazan. Advantages of NT over TPT have been demonstrated in various laboratories, *e.g.* (14).

When TPT was used by Perry and Cumming (13) in a quantitative study on rat adrenal homogenates, 5 mg. of ACTH injected subcutaneously 4 hr. before sacrifice caused a 39% increase in the activity of the dehydrogenase system when based on wet weight, and only a 12% increase on a total nitrogen basis. From the standard deviation given, the latter has

little significance. These results are in accord with those of the present study. Perry and Cumming also observed a similar result when the same dose of ACTH was administered in the same way each day for 7 days.

Suppression of adrenal activity by repeated injections of cortisone tended to produce higher activities per unit protein-nitrogen in the zonae glomerulosa and fasciculata, although the effect was considerably diminished on the wet weight basis (Fig. 3). Black and Speer (15) observed a similar increased activity in the mouse adrenal by visualization with TPT when cortisone was added *in vitro*. However, Perry and Cumming found a significant decrease in the activity of rat adrenal homogenates with TPT, both on the wet weight and total nitrogen basis, when the animals were subcutaneously injected with 5 mg. of cortisone daily for 7 days. The reason for the difference between this result and that given in Figure 3 may reside in the different animal treatments employed and the different kinds of samples, tetrazolium compounds and analytical techniques used. Bourne (16) mentioned that, although NT staining indicated increased activity in a variety of other rat organs following cortisone treatment, a decrease appeared in the adrenal. The reason for the discrepancy between the latter observation and the data in Figure 3 is not apparent.

The reduced activities found in the reticular and mixed reticular and medullary regions which accompany the atrophic effects induced in the adrenal by hypophysectomy (Fig. 4) are in accord with the reduced TPT staining in the reticularis observed by Zweifach *et al.* (12). These workers also reported less staining in the zona fasciculata. The highly variable effects of hypophysectomy on rat adrenal enzymes is far from clear. In all zones β-glucuronidase was found to increase (17), phenolsulfatase to decrease in the fasciculata and reticularis, and acetylcholinesterase to remain essentially unchanged (18). Rudolph and Olsen (19) found that glucose-6-phosphate dehydrogenase in whole gland homogenates changed little, while 6-phosphogluconate dehydrogenase was lowered, on a wet weight basis.

Acknowledgments

The authors are indebted to Miss M. Novotny and Mrs. F. Kvalnes for technical assistance.

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DETECTION OF MINUTE AMOUNTS OF ADRENOCORTICOTROPIC HORMONE BY THE EFFECT ON ADRENAL VENOUS ASCORBIC ACID[1]

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ABSTRACT

Adrenal venous blood was collected from hypophysectomized plateaued female rats immediately following intravenous injection of ACTH. The ascorbic acid concentration of the adrenal venous blood, of systemic blood, and of the adrenal gland before and after administration of ACTH was estimated.

The increase in adrenal venous ascorbic acid concentration during the period of cannulation (on the average, 9.0 min.) was related linearly to the log dose of ACTH over the range, 0.01 to 0.10 mU./100 gm., with a standard deviation(s) of 2.12, a slope (b) of 20.0, and an index of precision (λ) of 0.106. A biological assay method based on this response was proposed for the assay of ACTH in blood.

The absolute increase in adrenal venous ascorbic acid was significantly greater ($P < .05$) than the loss in adrenal ascorbic acid during the period of cannulation, suggesting that ACTH had increased the rate of biosynthesis as well as the rate of secretion of adrenal ascorbic acid.

A simplified technique for collection of adrenal venous blood employing an ordinary syringe and needle and applicable to young rats weighing as little as 150 gm. was developed. By this technique a marked increase in adrenal venous asorbic acid concentration was observed in blood samples collected for 60 sec., beginning 30 sec. after intravenous injection of 0.10 mU. of ACTH/100 gm.

THE detection and quantitative estimation of the minute concentration of adrenocorticotropic hormone (ACTH) in blood and other extrapituitary tissues are hampered by the lack of a sufficiently delicate biological assay method. For example, in our hands, the minimum effective dose (MED) of ACTH in the method based on reduction of adrenal ascorbic acid in hypophysectomized rats (1, 2) is approximately 0.25 milliunit (mU), while the ACTH concentration in the blood of the rat, even after elevation following adrenalectomy, is only about 0.03 mU./ml. (3).

Recently it has been reported that there is a marked increase in the concentration of ascorbic acid in the adrenal venous blood of the hypophysectomized rat following administration of ACTH (4, 5). Subsequently, we observed that this effect can occur under conditions in which there is

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only a minimal or even undetectable change in adrenal ascorbic acid concentration. We were therefore led to determine the effect of a range of very small doses of ACTH on adrenal venous ascorbic acid and to study simplifications in technique in order to provide the basis for a feasible biological assay method.

MATERIALS AND METHODS

Principal Experimental Procedure. The rats used in these experiments were plateaued females obtained commercially.[2] Females, rather than males, were used because male rats of the requisite size, 350–450 gm., were not available. In the technique used for cannulation of the adrenal vein, female rats of considerably smaller size could be used because of the larger adrenal veins, corresponding to the larger adrenals (per 100 gm. body weight) in the female sex.

The general health of the animals was only moderately good. Only 75% of 125 rats hypophysectomized survived the operation. One-third of the survivors were unsuitable for cannulation because of poor general condition or small adrenal veins. In an additional one-third of the survivors, the cannulation failed because of technical difficulties.

Routinely, three or four rats were hypophysectomized in preparation for an experiment on no more than two rats the following day. The operation of hypophysectomy was performed in the usual manner, using the parapharyngeal approach. The following day, 18 hours after hypophysectomy in most cases, the rats were weighed and anesthetized by an intraperitoneal injection of 6 mg. of sodium pentobarbital in 0.1 ml. of water. The time interval between the hypophysectomy and the experiment was slightly different for 5 of the 21 rats in the experiment, 12 hours for 3 rats, 15 hours for 1 rat, and 20 hours for 1 rat, without detectable influence on the results. The body weights of the rats ranged from 210 to 310 gm., with a mean of 241 gm. Occasionally, an additional amount of pentobarbital, usually not exceeding 3 mg., was necessary to provide adequate anesthesia.

About 5 min. after administration of the anesthetic, the rat was ready for the experiment. First, a sample of 1.0 ml. of systemic blood was collected by direct cardiac puncture. Within 2 to 5 min. following this procedure, a laparotomy was performed, and the right adrenal was removed and put aside in a moist place for later clean dissection and weighing. Heparin solution, 100 U./100 gm., body weight was then injected into a femoral vein. Immediately thereafter, a selected dose of ACTH in 0.1 ml. of acidified (pH2) physiological salt solution per 100 gm. was injected into the same vein. The total time interval between removal of the right adrenal and injection of ACTH was 2 to 5 min. Within 1 to 2 min. after injection of ACTH a fine glass cannula with collecting cup was inserted directly into the left adrenal vein with the aid of a dissection microscope. The blood was allowed to flow until 0.5 to 1.0 ml. had been collected. The time required, on the average, was 9.0 min. (standard deviation, 3.2 min.). Immediately after completion of the collection of adrenal venous blood, the cannulated left adrenal was removed. One min. later a second sample of 1.0 ml. of systemic blood was obtained by cardiac puncture.

The average time interval that elapsed between the collection of the two samples of cardiac blood was 17.4 min., with a standard deviation of 2.7. This relatively short time interval tended to minimize but did not completely avoid the gradual rise in apparent ascorbic acid concentration of the systemic blood that occurs in the rat during operative trauma, independently of the pituitary and adrenal (4, 5). The mean rise in ascorbic

[2] Sprague-Dawley, Inc., Madison, Wisconsin.

acid concentration in systemic blood during the interval between the collection of the two cardiac blood samples was 0.85 ± 0.23 μg./ml.[3] This rise was statistically significant at $P < .01$, but it was of no great magnitude, only 14% of the initial ascorbic acid concentration, which was 6.1 ± 0.20 μg./ml.

The sequence of procedures and the approximate time relations are summarized in Table 1.

Analytical Method for Ascorbic Acid. A semimicro modification of the method of Roe and Kuether (6) was used to determine the ascorbic acid concentration in the blood samples and adrenal glands. One ml. or less of whole blood was added immediately after collection to 3.0 ml. of 6% aqueous trichloroacetic acid (TCA) solution. Activated charcoal, fine, 0.15 gm., was added, and the mixture was shaken for 1 min., then allowed to stand for 5 min. The suspension was filtered through Whatman No. 42 filter paper, 7 cm. in diameter. To 1.0 ml. of the filtrate were added 1 drop of a 6% aqueous solution

TABLE 1. TIMING OF PROCEDURE FOR DETERMINATION OF EFFECT OF ACTH ON ADRENAL AND ADRENAL VENOUS ASCORBIC ACID

	Approximate time
	min.
Injection of sodium pentobarbital	−5
Collection of first sample of systemic blood	0
Laparotomy and removal of right adrenal	3
Injection of heparin	5
Injection of ACTH	6
Insertion of cannula into left adrenal vein	7
Collection of adrenal vein blood completed	16
Removal of left adrenal	16
Collection of second sample of systemic blood	17

of thiourea, and 0.25 ml. of a 2.0% solution of 2,4-dinitrophenylhydrazine in 9N sulfuric acid. The mixture was shaken and then incubated in a water bath at 37° C for 3 hours. At the end of the incubation the tubes were cooled in an ice bath, and 1.25 ml. of 85% sulfuric acid was added with mixing. After standing for 15 min. the ascorbic acid in each sample was estimated by measurement at 515 mμ in a Beckman model DU spectrophotometer in comparison with standard solutions prepared similarly.

For measurement of adrenal ascorbic acid concentration, each gland, after dissection and weighing, was crushed in a conical glass homogenizer and mixed with 4.0 ml. of 4% aqueous TCA. The homogenate was treated with charcoal and filtered as above. One ml. of the filtrate was diluted to 10 ml. with 4% TCA, and one ml. of this diluted filtrate was treated exactly the same as the blood filtrate.

The ACTH Preparations and Their Biological Assay. The ACTH preparations used in the experiments were prepared for human intravenous use and were obtained from the Upjohn Co. Both preparations were reassayed by us against the U.S.P. Corticotropin Reference Standard, by the so-called Munson modification of the assay method of M. A. Sayers *et al.* (1), using hypophysectomized rats and intravenous injections. Our assay of Lot. No. 10,918–3 was in good agreement with the statement on the label, showing a potency of $109\%^{\times}_{\div}1.18$[4] of the labeled value. Our assay of Lot No. HD482 DL showed a potency $201\%^{\times}_{\div}1.18$ of the labeled value, and, accordingly, it was used as if it were twice as potent as labeled.

[3] In this paper, in all cases, the value following the ± sign is the standard error.

[4] The symbol, $^{\times}_{\div}$, signifies that the factor following it should be multiplied by and divided into the estimate of potency in order to obtain the limits of the standard error (7).

RESULTS

Effect of ACTH on Adrenal Venous Ascrobic Acid Concentration. The relation of the increase in adrenal venous ascorbic acid concentration in hypophysectomized rats to the dose of ACTH, administered intravenously as described above, is shown in Figure 1. The log dose-response curve is linear between 0.01 and 0.1 mU. By t-test a statistically significant effect above the controls was obtained with a dose of only 0.01 mU. However, because of the small number of animals compared and the position of the extrapolated log dose-response curve, a safe estimate of the MED would be between 0.01 and 0.02 mU., rather than definitely at the lower level.

For the four dose levels associated with the log dose-response curve in Figure 1, the standard deviation, s, was 2.12, and the slope, b, was 20.0, giving an index of precision, λ, of 0.106. The experiment, therefore, promises very well for a precise biological assay method planned along similar lines. However, the statistics were based on data from only 14 rats, and a more reliable evaluation must await the accumulation of a larger body of data.

The limited amount of data from doses of ACTH larger than 0.1 mU. suggests that the maximum effect was obtained at 0.1 mU., and that higher doses may produce somewhat less than maximal effects.

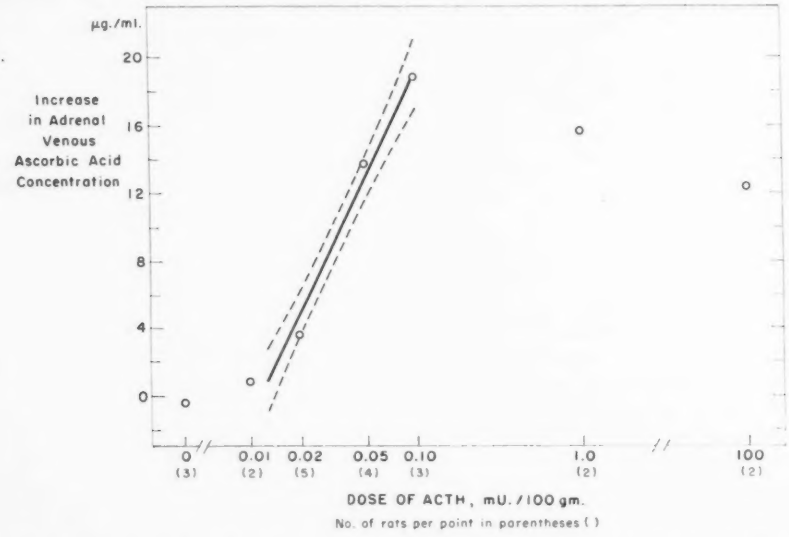

FIG. 1. Increase in adrenal venous ascorbic acid concentration (concentration in adrenal venous blood minus mean concentration in systemic blood) following administration of different doses of ACTH. The solid black line represents the linear log dose response curve between 0.01 and 0.10 mU. of ACTH/100 gm., calculated by the method of least squares. The dashed lines represent the standard error.

Effect of Low Doses of ACTH on Adrenal Ascorbic Acid Concentration in Hypophysectomized Plateaued Female Rats. The MED of ACTH in the classical biological assay method, whether conducted by the original procedure of M. A. Sayers *et al.* (1) or by our modification (2), is approximately 0.25 mU./100 gm. This value was obtained in young hypophysectomized male rats weighing from 100 to 160 gm. Additional observations were made in order to determine whether the MED in the proposed new method was lower than in the classical method because of the response measured or merely because of the animals and conditions used for the test.

An ACTH assay was conducted by the usual procedure in 15 female rats from the same lot used for cannulation of the adrenal vein. The rats had been hypophysectomized 22 hours previously and at the time of the assay weighed 200–230 gm. Three different dose levels of ACTH (U.S.P. Corticotropin Reference Standard) and acidified (pH2) physiological salt solution as a control were administered by intravenous injection. The right adrenal was removed one hour after injection and analyzed for ascorbic acid. The left adrenal was removed from one rat of each group just before the injection, thus providing a second set of control data. The results are given in Table 2. They show that a dose of 0.05 mU./100 gm. had no significant effect on adrenal ascorbic acid concentration one hour after injection and that the MED under these test conditions was approximately 0.2 mU./100 gm.

The changes in the adrenal ascorbic acid concentration during the experiments with adrenal vein cannulation were also determined. In these experiments the time interval between injection of ACTH and removal of the second adrenal gland was much shorter, only 10 min. as compared with one hour in the previous experiment. The results are given in Table 3. Even at the higher dose levels the change in adrenal ascorbic acid concentration in the time interval used was relatively small. These test conditions are apparently quite unsuited for biological assay. However, a small but significant fall in adrenal ascorbic acid concentration occurred at a dose level of only 0.05 mU./100 gm. A possible explanation for the discrepancy in MED between the two experiments is that the effect of small doses of ACTH is relatively brief, and, although it is evident 10 min. after injection there is a return to the control level before the lapse of one hour.

Relation between Amount of Ascorbic Acid Lost from the Adrenal and Amount Recovered in the Venous Effluent. The experiment on the effect of ACTH on adrenal venous ascorbic acid was designed so that the amount of ascorbic acid lost from the adrenal during collection of the adrenal venous blood could be estimated. The amount of ascorbic acid in the left adrenal *before* injection of ACTH was estimated by multiplying the weight of the adrenal by the concentration of ascorbic acid in the right (control) adrenal on the assumption, only approximately correct, that the ascorbic acid con-

TABLE 2. RESULTS OF ACTH ASSAY ON HYPOPHYSECTOMIZED FEMALE RATS OF 200–230 GM. BODY WEIGHT

Treatment	Adrenal ascorbic acid concentration	
	Mean	Individual values
	mg./100 gm. adr. wt.	mg./100 gm. adr. wt.
0.05 mu. ACTH	451	446[a], 434, 478, 445
0.20 mu. ACTH	402	427[b], 417, 362, 400
0.80 mu. ACTH	349	367[c], 333, 340, 356
Control solution	445	442[d], 417, 477
Control adrenals	446	443[a], 446[b], 458[c], 438[d]

The doses of ACTH were given per 100 gm. body weight. A control adrenal was taken from one rat in each treatment group immediately before the injection of ACTH or the control solution. The superscript letters identify the paired values.

centration of the two adrenals before ACTH administration was identical. Subtraction from this value of the amount of ascorbic acid found by analysis to be present in the left adrenal *after* injection of ACTH gave the best possible estimate of the amount of ascorbic acid lost from the adrenal during cannulation. In Table 4, the resulting values are compared with the corresponding values for increase in adrenal venous ascorbic acid, which were obtained from the volume of blood collected and the increase in adrenal venous concentration over the mean systemic blood concentration.

As shown in Table 4, there was an increased amount of ascorbic acid in the venous effluent in every one of the 18 rats in the experiment that were given ACTH. (The variability of the values is greater than that shown in Fig. 1 because of the differences in volume of blood, which did not enter into the data of Fig. 1.) In 6 of the 18 rats there was an apparent but not necessarily true gain in adrenal ascorbic acid following ACTH. There was

TABLE 3. FALL IN ADRENAL ASCORBIC ACID CONCENTRATION IN HYPOPHYSECTOMIZED FEMALE RATS DURING CANNULATION OF THE ADRENAL VEIN AND APPROXIMATELY 10 MIN. AFTER INJECTION OF ACTH

Dose of ACTH	Fall in adrenal ascorbic acid concentration*	
	Mean	Individual values
mu./100 gm. body wt.	mg./100 gm. adr. wt.	mg./100 gm. adr. wt.
100	50	39, 61
1.0	44	15, 73
0.10	25	−2, 26, 52
0.05	31	37, 35, 22, 30
0.02	− 7	−4, −27, 15, −17, −2
0.01	− 0.5	2, −3
0.00	−11	−30, −10, 8

* Adrenal ascorbic acid concentration of right (control) adrenal minus that of left (stimulated) adrenal. Negative values signify an apparent rise in adrenal ascorbic acid concentration.

an apparent excess of ascorbic acid found in the venous effluent over that lost from the gland in 14 of the 18 rats. The mean excess adrenal venous ascorbic acid and its standard error are given in the table. By the one-tailed t-test, this "excess" was barely significant statistically at P.05. The many assumptions inherent in the calculations and the smallness of the excess ascorbic acid incline us to view this result with caution. Additional experiments utilizing a somewhat different approach to the problem are in

TABLE 4. CHANGES IN ADRENAL ASCORBIC ACID AND ADRENAL VENOUS ASCORBIC ACID DURING THE COLLECTION OF A SAMPLE OF ADRENAL VENOUS BLOOD BY CANNULATION

Dose of ACTH	Loss of adrenal ascorbic acid*	Increase in adrenal venous ascorbic acid	Adrenal venous ascorbic acid in excess of loss from adrenal†
mu./100 gm. body wt.	μg.	μg.	μg.
	10.5	16.5	6.0
100	15.7	9.1	−6.6
1.0	3.6	5.5	1.9
	21.9	20.3	−1.6
0.10	−0.6	10.9	11.5
	6.4	8.0	1.6
	14.4	8.4	−6.0
0.05	9.3	7.4	−1.9
	11.7	15.1	3.4
	6.8	13.5	6.7
	11.0	11.5	0.5
0.02	−1.1	1.4	2.5
	−6.9	2.8	9.7
	3.6	1.9	−1.7
	−5.6	2.4	8.0
	−0.6	1.3	1.9
0.01	0.6	0.6	0.0
	−0.6	0.4	1.0
			Mean 2.05 ± 1.16

* A negative value in this column denotes an apparent gain rather than a loss of adrenal ascorbic acid.

† A negative value in this column indicates the extent to which the increase in adrenal venous ascorbic acid was less than the loss in adrenal ascorbic acid.

progress to test the interesting hypothesis proposed earlier (8) that ACTH stimulates the biosynthesis of adrenal ascorbic acid as well as its secretion.

Development of an Improved Method for Collecting Adrenal Venous Blood from the Rat. The method of collection of adrenal venous blood used in the experiment described above is very exacting technically. The special fine glass cannulas with collecting cup are difficult to make and are rather perishable. They can be inserted into the adrenal vein effectively only after long experience and the attainment of special skill. Large rats must be used. A new extremely simple and effective method has been devised by one of

us (W.T.). It employs an ordinary 0.5 ml. hypodermic syringe fitted with a 26 or 27 gauge needle and can be used in male rats weighing as little as 150 gm. The needle is inserted into the renal vein just caudal to the juncture with the adrenal vein. The dose of ACTH may be injected into the renal vein at this time. Without withdrawal, the needle is then gently worked into the adrenal vein, occluding it. The needle is turned so that the bevel faces the lumen of the vein. The plunger of the syringe is then slowly pulled back allowing the adrenal venous blood to flow into the syringe. By continual observation through the dissection microscope the rate at which the plunger is retracted can be regulated just to keep pace with the rate of flow.

In a preliminary experiment in which the new technique was used on hypophysectomized male rats weighing from 180–190 gm., a marked increase in adrenal venous ascorbic acid concentration was observed in samples collected for 60 sec., beginning 30 sec. after intravenous injection of ACTH, 0.10 mu./100 gm.

DISCUSSION

The analytical method that was used for ascorbic acid in this work (6) is not completely specific (9); dehydroascorbic acid, diketogulonic acid, and reductones interfere. Therefore, the true identity of the constituents of adrenal venous blood that behave analytically like ascorbic acid has not been determined with certainty. That ascorbic acid is the major component of the increase, however, is supported by data on the recovery of labeled ascorbic acid. Salomon has reported (10) that in rats previously injected with ascorbic acid-1-C^{14}, from 62 to 82% of the radioactivity in the adrenal venous plasma after administration of ACTH could be identified as ascorbic acid. Although further investigation of the composition of the total apparent increase in adrenal venous ascorbic acid is in order, the usefulness of the proposed biological assay method does not depend on an unequivocal identification of ascorbic acid in the adrenal effluent.

A further study of the time relations between the adrenal secretion of ascorbic acid and of cortical steroids should help to clarify the possible functional association of the two processes. Our results in the hypophysectomized rat have shown an increase in adrenal venous ascorbic acid within 60 seconds after administration of ACTH. Nelson and Hume (11) demonstrated that in the hypophysectomized dog the increase in adrenal venous 17-hydroxy cortical steroids also occurs very rapidly, within 2 to 4 min. after injection of ACTH. On the other hand, Slusher and Roberts (5) have concluded that the increased secretion of steroids by the rat adrenal after ACTH administration is much slower to appear than the increase in ascorbic acid.

If the very small MED and the precision of our experiments can be maintained in the proposed new assay method the method should provide

more reliable assays of ACTH in blood than have heretofore been possible (12). Two or more dose levels of an unknown could be tested on adequate numbers of animals without the need for impractically large samples of blood. For example, 2 to 4 ml. of plasma from the average patient with Addison's disease, which contains, according to the best available estimate of plasma ACTH concentration, 0.084 mU./ml. (13), should be an adequate amount for an entire quantitative assay, and it might even be feasible to assay for ACTH in normal plasma without prior fractionation (14).

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BLOOD ACTH: EFFECTS OF ETHER, PENTOBARBITAL, EPINEPHRINE AND PAIN[1]

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ABSTRACT

The exogenous test for ACTH release, analysis of blood ACTH in a recipient hypophysectomized rat, provides information on the rapid changes in blood ACTH which may follow application of a stimulus. The endogenous test, analysis of ACTH release by adrenal ascorbic acid depletion in the stimulated animal, fails to uncover these dynamic changes.

A painful stimulus markedly elevates blood ACTH within a few minutes of application. Elevated blood levels persist during 30 minutes of intermittent painful stimulation.

Ether first excites, then inhibits ACTH release. Pentobarbital exhibits only a depressant action. The excitatory action of ether is blocked by decerebration and by destruction of the median eminence area of the hypothalamus. The excitatory action of epinephrine is manifest in the decerebrate but not in the median eminence lesion rat. Since ether and pentobarbital depress the brain stem reticular system, it is reasonable to speculate that the action of these two agents in inhibiting ACTH release involves this multisynaptic conduction system.

The reduction by pentobarbital of blood ACTH below the resting level characteristic of undisturbed adrenalectomized rats suggests that stimuli encountered in everyday existence such as light, sound and touch, exert a tonic action on the pituitary to maintain a low rate of release of ACTH.

INHALATION of ether has been demonstrated to induce release of ACTH from the pituitary (1), whereas injection of sodium pentobarbital is generally followed by at least partial suppression of ACTH release in the rat (2, 3). Both these anesthetics have been demonstrated by French *et al.* (4) to selectively depress the conduction of impulses over the medial multisynaptic reticular activating system but not the lateral paucisynaptic classical pathway of the brain stem. The apparent lack of parallelism between the action of ether on the reticular activating system and its action on the release of ACTH prompted us to re-examine the effects of ether inhalation on pituitary release of ACTH with a technique designed

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to uncover instantaneous changes in blood ACTH levels. Sodium pentobarbital, epinephrine and cutaneous electric shock were examined for comparative purposes.

METHODS

ACTH release following ether inhalation was determined by anesthetizing rats (120 to 150 gm.) with ether for 60 minutes. At the end of this time, both adrenals were removed and each adrenal was analyzed for ascorbic acid. A control group of untreated rats was decapitated and adrenal ascorbic acid determined. This procedure is designated the *endogenous test* since ACTH release is assessed in the animal in which it is elicited.

A second procedure, the *exogenous test,* was also used to evaluate ACTH release. Changes in the level of blood ACTH were determined by injecting blood from experimental donor rats (250 to 400 gm.) into 24-hour hypophysectomized recipient rats (90 to 130 gm.). The hypophysectomized recipient rat was anesthetized with ether and the left adrenal removed. Blood was drawn into a heparinized syringe from either the abdominal aorta or the jugular vein of the experimental donor rat and quickly transferred to a femoral vein of the recipient hypophysectomized rat. Less than 60 seconds elapsed between the initiation and completion of the blood transfer. One hour later the recipient animal was re-anesthetized and the right adrenal removed. The animal was then sacrified and the sella examined for pituitary remnants. The blood ACTH level of the donor animal is expressed as the difference in ascorbic acid concentration (mg. per 100 gm. adrenal wet weight) between the left and right adrenal of the hypophysectomized recipient animal. Since adrenal ascorbic acid depletion in the hypophysectomized rat is not a rectilinear function of ACTH injected, it should be noted that a twofold change in adrenal ascorbic acid depletion indicates a more than twofold change in blood ACTH level in the donor rat.

Resting levels of blood ACTH in the non-anesthetized animal were obtained by gently removing a donor animal from its cage and quickly placing a large surgical clamp about its neck to prevent exchange of blood between the head and the trunk. Blood was removed from the abdominal aorta and assayed in hypophysectomized recipient rats

Rats were painfully stimulated in a box with a metal grid floor; condensor discharges (Harvard Stimulator Model 935-A, 250 volts, 120 pulses per second) were applied across the grid for 3 seconds of each minute. The grid was intermittently moistened with a 1% sodium chloride solution to insure good contact with the paws of the rat. Under these conditions the D-C resistance of the rat to the current flow was 20,000 to 200,000 ohms. A neck clamp was applied after a predetermined time of stimulation and a sample of blood drawn from the abdominal aorta.

Brain stem transection was performed as follows. The animals were anesthetized with ether and the calvarium exposed. A hole was drilled with a No. 8 dental burr 3 to 5 mm. lateral to the midline and just rostral to the posterior suture. The burr hole was located dorsal to the junction of the inferior and superior colliculi. The shaft of a No. 2 dental probe, blunted at the end with solder, was introduced through the burr hole and drawn down one side of the brain case, across the floor and up the other side. Transection was through the mid to upper level of pons. The section passed 1 to 3 mm. caudal to the most caudal aspect of the pituitary. Completeness of section was checked by gross inspection of the formaldehyde fixed brains. In sham decerebrate animals, a hole was drilled in the skull under ether anesthesia. All operated animals were allowed to recover from ether anesthesia 70 to 200 minutes before application to the test stimulus.

In some rats (250 to 300 gm.), lesions were made in the hypothalamic area with a

high frequency alternating current. The electrode was placed by a stereotaxic instrument. The lesioned rats used in the study exhibited marked diabetes insipidus when tested six days after lesioning. Seven days after lesioning they did not respond to ether anesthesia and unilateral adrenalectomy with adrenal ascorbic acid depletion in the remaining adrenal. The adrenalectomized rats were injected daily with 0.1 mg. desoxycorticosterone acetate and provided with tap water to drink. Three or more weeks after bilateral adrenalectomy, 2 ml. of jugular vein blood were withdrawn for ACTH assay in recipient hypophysectomized rats. All lesioned rats were tested two or more times with this procedure.

No actue reactions attributable to blood incompatibilities were noted in the recipients. All rats were males obtained from the Holtzman Company.

RESULTS

Adrenalectomy and Blood ACTH. No measurable quantity of ACTH was detected in the blood of acutely decapitated rats with intact adrenals; furthermore, exposure to ether for 5 minutes did not induce a detectable increase in blood ACTH (Table 1). In contrast, rats bilaterally adrenalectomized three or more weeks prior to use and given 1% sodium chloride to drink exhibited measurable quantities of ACTH in the blood following acute decapitation. Ether anesthesia of 5 minutes duration was associated with a marked elevation in the level of blood ACTH in the adrenalectomized rat (Table 1). The chronically adrenalectomized rat thus represents a suitable test animal in which to evaluate the influence of various factors on blood ACTH levels in the absence of corticosteroids which are known to inhibit ACTH release, and the absence of the major source of epinephrine which is known to promote ACTH release.

Pain and Blood ACTH. A painful stimulus elevates blood ACTH levels in the unanesthetized adrenalectomized rat. After five minutes of repetitive electric shock applied to the paws, the blood ACTH levels were markedly elevated. After 30 minutes of intermittent stimulation, the blood ACTH levels remained elevated (Table 2). Repeated application of a painful stimulus is capable of evoking a sustained elevation of blood ACTH level in the unanesthetized adrenalectomized rat.

Pentobarbital and Blood ACTH. An anesthetic dose of sodium pentobarbital significantly decreases ($P<.05$) the resting level of blood ACTH. Samples taken at 5 and 15 minutes after administration of the anesthetic

TABLE 1. ADRENALECTOMY AND BLOOD ACTH

Donor rat	Stimulus	No. of rats	Adrenal ascorbic acid depletion in recipient rat,* mg./100 gm.
Intact	None (resting level)	11	6 ± 9†
Intact	Ether (5 minutes)	10	−11 ± 8
Adrenalectomized	None (resting level)	9	42 ± 9
Adrenalectomized	Ether (5 minutes)	10	117 ± 8

* All recipient hypophysectomized rats received 4 ml. of donor blood (from aorta).
† Mean and standard error.

TABLE 2. EFFECTS OF PAIN, PENTOBARBITAL AND ETHER ON BLOOD ACTH

Treatment	Adrenal ascorbic acid depletion in recipient rat,‡ mg./100 gm.					
	Resting level	2 min.	5 min.	15 min.	30 min.	60 min.
Electric shock	28 ± 7† (24)*	—	104 ± 11 (16)	—	83 ± 10 (20)	—
Pentobarbital anesthesia	42 ± 9 (9)	—	−5 ± 16 (5)	12 ± 7 (7)	—	—
Ether anesthesia	42 ± 9 (9)	109 ± 6 (21)	117 ± 8 (10)	63 ± 12 (8)	41 ± 6 (47)	12 ± 9 (9)

* Number of rats in parentheses.
† Mean and standard error.
‡ All recipient hypophysectomized rats received 4 ml. of donor blood (from aorta).

agent to adrenalectomized donors exhibited no detectable quantities of ACTH (Table 2).

Ether, Epinephrine, and Blood ACTH. Intact rats anesthetized with ether for 60 minutes exhibited adrenal ascorbic acid depletion as compared to untreated intact rats (6 control untreated rats, 452 ± 39 mg. ascorbic acid per 100 gm. adrenal; 6 ether anesthetized rats, 219 ± 8 mg. ascorbic acid per 100 gm. adrenal). The endogenous test reveals only a stimulating action of ether on ACTH release. In contrast, the response to ether is biphasic when measured by the exogenous test. In adrenalectomized rats, sacrificed 2 minutes after the start of ether anesthesia, blood ACTH was markedly increased over the control level (Table 2). After 5 minutes of anesthesia, blood ACTH remained elevated. Blood ACTH in animals maintained under ether anesthesia for 15 minutes was significantly reduced from peak levels; after 30 minutes of continuous anesthesia it had returned to pre-anesthetic levels and at 60 minutes, was significantly less ($P < .05$) than the resting level. The response to ether is characterized by an initial excitatory phase and a subsequent inhibitory phase.

TABLE 3. BLOOD ACTH IN DECEREBRATE AND HYPOTHALAMIC LESIONED ADRENALECTOMIZED RATS

Exp. no.	Donor rat	Stimulus	No. of rats	Adrenal ascorbic acid depletion in recipient rat,* mg./100 gm.
1	Sham decerebrate	None (resting level)	6	24 ± 9†
2	Sham decerebrate	Ether (2 minutes)	9	116 ± 6
3	Decerebrate	None (resting level)	4	11 ± 20
4	Decerebrate	Ether (2 minutes)	13	29 ± 11
5	Decerebrate	Epinephrine 10 μg. infused i.v. over 5 minutes	8	105 ± 10
6	Decerebrate	Nor-epinephrine 10 μg. infused i.v. over 5 minutes	7	82 ± 10
7	Lesioned	Ether (5 minutes)	27	−4 ± 6
8	Lesioned	Epinephrine 10 μg. infused i.v. over 5 minutes	13	32 ± 8

* Recipient hypophysectomized rats received 4 ml. blood from donor decerebrate rats or 2 ml. blood from donor lesioned rats.
† Mean and standard error.

The inhibitory phase is not associated with cardiovascular collapse in the adrenalectomized donor. Blood pressure was measured in ether-anesthetized adrenalectomized and intact rats with a mercury manometer connected to the carotid artery. After 15 minutes of ether anesthesia, the average blood pressure in 14 adrenalectomized rats was 115 mm. Hg (range 90 to 141) and at 30 minutes, 111 mm. Hg (range 90 to 140). The average values in four intact rats were 111 (range 102 to 120) and 111 (range 102 to 118) at 15 and 30 minutes, respectively. The average rectal temperature after 30 minutes of ether anesthesia in 5 adrenalectomized rats was 36.9° (range 36.1 to 37.4), and in 5 intact rats, 37.1° (range 36.5 to 37.5).

The inhibitory phase of the pituitary response to ether is not accompanied by exhaustion of pituitary stores of ACTH. Five adrenalectomized rats were sacrificed at 2 minutes and 5, at 30 minutes after the start of ether anesthesia. The pituitaries of each group were pooled and assayed (5). Two separate experiments were conducted. In one, the pituitary content of ACTH after 30 minutes of ether anesthesia was 1.3 (standard error = 0.5) and in another, 1.9 (standard error = 0.6) times the content after 2 minutes of ether anestheisa. Furthermore, the responses exhibited by the hypophysectomized test rats indicated that the absolute quantity of ACTH in the pituitary of the adrenalectomized rat during ether anesthesia is 100 to 250 milliunits and approximately equal to that in the piutitary of the non-stimulated adrenalectomized rat (5).

The excitatory phase of ether was almost completely blocked by midbrain transection (decerebration) (Expts. 2 and 4, Table 3). Such an operative procedure does not render the animal incapable of responding to all stimuli known to initiate ACTH release, since epinephrine and nor-epinephrine elevated blood ACTH levels in the decerebrate preparation (Expts. 5 and 6, Table 3). The excitatory phase of ether anesthesia is probably due to nervous impulses arising caudal or peripheral to the upper level of pons. Some of these impulses may originate in the respiratory epithelium and follow afferent paths to the bain stem. A lesion in the area of the median eminence of the hypothalamus almost completely blocked the excitatory action of epinephrine and completely blocked the excitatory action of ether on the pituitary (Expts. 7 and 8, Table 3). A locus of action of epinephrine in eliciting ACTH release appears to be an area between upper level of pons and the median eminence.

DISCUSSION

The endogenous technique for evaluating ACTH release reveals only the excitatory component of action of ether on the adenohypophysis. Fluctuations of blood ACTH levels with time cannot be uncovered by this technique. A phasic response of the pituitary to ether is detected with the exogenous technique because serial sampling of blood is employed. A distinct advantage of the exogenous technique is its ability to detect decrease

as well as increases in blood ACTH titers at any time after stimulus application. Also, since in the exogenous test, ACTH is assayed in a standardized preparation (the hypophysectomized rat), the problem of alteration in the sensitivity of the adrenal of the assay animal to ACTH, as may occur in the chronic lesion animal, is eliminated.

The difference in blood ACTH levels between intact and adrenalectomized rats is again confirmed (5). Not only do the adrenalectomized rats exhibit readily detectable resting blood ACTH levels, but following a stimulus, the blood levels exhibit pronounced elevation. Only under exceptional circumstances are detectable amounts of ACTH found in the blood of intact rats when an exogenous technique is employed (5, 6).

We assume that the neural components involved in excitation of the pituitary act qualitatively the same in the intact and in the adrenalectomized rat. The quantitative difference is the consequence of the inhibitory influence of the corticosteroids on the nervous system, the adenohypophysis or both.

The excitatory phase seen with ether is attributable to the events occurring immediately preceding the development of the anesthetic state. The rat is abruptly placed in an ether jar and exposed to irritating ether vapor. The hypothalamus and pituitary may be activated by impulses originating in the respiratory epithelium and ACTH is released. With the onset of the anesthetic state fewer impulses reach the hypothalamus and ACTH release declines. In contrast, throughout a 30 minute period of intermittent painful stimulation in the unanesthetized rat, the rate of release of ACTH remains relatively high. Since the pituitary is capable of a sustained high level of activity, the inhibitory phase of ether action is most likely a consequence of the inhibitory influence of this agent on the brain. This conclusion assumes that ether does not influence the degradation and excretion of ACTH.

The excitatory component of ether, but not of epinephrine is eliminated by brain stem transection at the upper level of pons. Elimination of the excitatory action of epinephrine by a lesion in the median eminence area (7) (Table 3) indicates that this drug has a locus of action somewhere between upper level of pons and the median eminence. Of interest in this connection is the demonstration by Porter (8) that epinephrine has a direct stimulating action on the posterior hypothalamus although Sutin and Clemente (9) have not confirmed this observation.

The fact that ether as well as pentobarbital inhibits release of ACTH from the adenohypophysis is in harmony with the known depressant action of these agents on the nervous system. Since their action is most pronounced on the brain stem reticular system (4), it is reasonable to speculate that these agents are inhibiting components in this multisynaptic system which are necessary for excitation of the pituitary. Sawyer (10) has proposed that morphine, chlorpromazine and pentobarbital inhibit the pitui-

tary by depressing the reticular activating system. Neuro-anatomical support for this thesis has been presented by Nauta and Kuypers (11) who have described connections between midbrain tegmental nuclei and the hypothalamus. It is not to be implied that the reticular activating system is the only path from the periphery to the hypothalamus which subserves ACTH release.

An important implication follows the demonstration that ether and pentobarbital decrease the resting level of ACTH in the blood of the adrenalectomized rat. Stimuli encountered under normal environmental conditions, e.g., light, sound and touch, would appear to exert a tonic action on the pituitary. Stimuli of greater intensity, e.g., pain, trauma, toxic agents, increase ACTH release over a tonic level of elaboration. The fact that an adrenalectomized rat with a lesion in the median eminence exhibits no detectable quantity of ACTH in the blood during the time when the excitatory response to ether is usually seen (Table 3) may be accepted as evidence that under resting conditions the level of hormone in the blood is also nondetectable. This may be interpreted to mean that destruction of the "final common path" blocks the tonic action of the nervous system on the pituitary. The diurnal variation in pituitary-adrenal function proposed by Halberg (12) may have as its basis a rhythmic tonic influence of the reticular activating system on the pituitary. The elimination of diurnal change in peripheral blood steroids following morphine administration (13) may be a consequence of the suppression of this rhythmic tonic influence of the reticular actvating system.

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THE EFFECT OF DIETARY FAT ON THE UTERINE WEIGHT RESPONSE OF IMMATURE MICE TO ORAL STILBESTROL

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ABSTRACT

The effect of dietary fat on the uterine weight of the immature mouse and the uterine weight response to stilbestrol added to the diet has been studied. Increasing the fat content of either beef or chicken lean tissue diets results in greater growth of the mouse as measured by an increase in body weight, and also in an increase in the uterine weight. The increase in uterine weight is proportionally greater than the increase in body weight. On the other hand, the uterine weight response to added stilbestrol in the diet of the mouse is decreased as the fat content is increased. The significance of these findings in the interpretation of estrogenic assays based on the mouse uterine weight method is discussed.

THE increasingly widespread use of stilbestrol to promote the rate of growth of steers and to fatten chickens has raised the question of the contamination of the edible tissues of treated animals with the drug. For the past two years, this laboratory has been conducting bioassays of such animal tissue to determine whether stilbestrol residues exist in them (1, 2, 3). The bioassay method, described in detail in another communication (4), consists of feeding the tissue to immature female mice for seven days and using the change in uterine weight as a criterion of response.

During the conduct of these assays, it was observed that the rate of growth of the uterus of the immature mouse varies with the diet (1). This was particularly noticeable when fatty tissue diets were fed. This observation prompted us to study the influence of fat on the uterine weight response to oral stilbestrol to enable us to interpret correctly bioassays designed to measure the amount of estrogenic residues in the edible tissues of stilbestrol-treated animals.

MATERIALS AND METHODS

The tissues used in this study were obtained from control animals which had not been treated with stilbestrol. Standard mouse laboratory diet (Purina chow) was mixed with the ground tissue in the proportion of 10 parts of laboratory diet to 100 parts of tissue. The diets were then rolled into six-gram patties and frozen. Each mouse received one six-gram patty per day, or a total of 42 grams during the seven-day feeding period. Before feeding each day, the unconsumed portion of the previous day's diet was sepa-

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rated from fecal contaminants and weighed. Although some weight loss occurred due to drying during the 24-hour feeding period, it is believed that the estimations of total food consumed are of sufficient accuracy to make valid comparisons. The mice were housed in individual cages and records of initial and final body weights were kept. The mice were sacrificed on the eighth day of the experiment, and their uteri were weighed without fixation.

The estimation of the total fat intake was calculated from the total food intake and the per cent of fat in the diet as determined by ether extraction. To facilitate the ether extraction, the tissues were ground with sea sand and anhydrous sodium sulfate.

The diets prepared from steer muscle and steer caul fat were fed raw. Diets prepared from chicken muscle, which consisted of both leg and breast meat, were cooked in an autoclave at 15 pounds pressure for 20 minutes. The chicken fat was obtained by rendering the fat from the skin of the chickens at 155° C for two hours. When stilbestrol was added to the diets, it was added in the form of an alcoholic solution of such concentration that each 100 grams of the diet received 1 ml. of the solution.

Weanling 21-day old female mice of the White Swiss General Purpose Strain of the National Institutes of Health, Bethesda, Maryland, were used.

RESULTS AND DISCUSSION

Table 1 shows the design of the experiment and the results obtained with mixed beef muscle and fat diets. In addition to the basic lean muscle diet, three other diets were prepared in which increasing amounts of lean muscle were replaced with ground beef fat. Each of these four diets was divided in half, the stilbestrol was added to one of the halves at a concentration of 10 parts per billion (0.010 μg./gm.). The resulting eight diets were then fed to groups of six mice each. The data from two identical runs were combined, statistical analysis of the data having shown that there was no significant difference between runs.

It will be noted that as the fat content of the diet increased, the body weight and the uterine weight of the mice also increased. However, the uterine weight increase was not proportional to the body weight increase as shown by an increase in the log ratio of the uterine weight to the final body weight. When these same four diets were fed with the addition of 10 parts per billion of stilbestrol, the body weights of the mice increased as the fat content of the diet increased as before, but there was a decrease in the uterine weight response to stilbestrol as the fat content of the diet was increased. Part of the decreased uterine weight response to added stilbestrol was due to the fact that there was less food consumed and, therefore, the total dose of stilbestrol was smaller. However, the last column of the table shows that the response per microgram of stilbestrol consumed was decreased as the fat content of the diet increased. In our study of the dose response relationship between oral stilbestrol and mouse uterine weight at low dosage levels, we have established that linearity is achieved if dose is plotted versus the log of the ratio of the uterine weight to the final body weight.

In Table 2 we have confirmed with chicken tissue diets that dietary fat

TABLE 1. EFFECT OF PROPORTION OF FAT AND LEAN TISSUE IN BEEF TISSUE DIETS ON THE BODY WEIGHT AND THE UTERINE WEIGHT RESPONSE TO STILBESTROL IN THE MOUSE

Composition of diet			DES in diet, ppb	No. of mice	Mean total food intake, gm.	Calc. total fat intake, gm.[b]	Calc. total DES intake, μg.	Mean initial body weight, gm.	Mean final body weight, gm.	Mean uterine weight, mg.	Mean log ratio[a]	Mean log ratio: treated less control	Change in log ratio per μg. of DES intake
Fat gm.	*Lean*, gm.	Mouse diet, gm.											
0	100	10	0	11	36	1.6	0.00	9.3	10.2	7.1 ± 0.7[c]	0.84 ± 0.04[c]	—	—
10	90	10	0	11	32	4.0	0.00	9.4	13.6	13.3 ± 1.5	0.96 ± 0.04	—	—
20	80	10	0	12	27	5.5	0.00	9.0	14.2	17.5 ± 2.6	1.05 ± 0.05	—	—
30	70	10	0	12	23	6.6	0.00	9.0	15.3	20.0 ± 2.8	1.08 ± 0.04	—	—
0	100	10	10	12	38	1.7	0.37	8.8	10.3	52.8 ± 2.9	1.69 ± 0.02	0.85	2.3
10	90	10	10	12	32	4.0	0.32	9.2	12.9	48.7 ± 3.4	1.57 ± 0.02	0.61	1.9
20	80	10	10	12	28	5.7	0.27	9.2	14.6	38.1 ± 3.6	1.40 ± 0.04	0.35	1.3
30	70	10	10	10	22	6.4	0.22	9.8	15.1	28.5 ± 4.4	1.23 ± 0.06	0.15	0.7

[a] Ratio = (Uterine Weight in mg./Final Body Weight in gm.) × 10.
[b] Based on a determination of ether-extractable solids: Per cent fat found for the four diet compositions were 4.59, 12.55, 20.60, and 28.71, respectively.
[c] Standard error of the mean.

affects the uterine weight of mice and that the uterine weight response of the mouse to oral stilbestrol is decreased as the fat content of the diet is increased. In this experiment, the fat content of the diets is less than in the beef fat experiment. The residues of unconsumed food were too small for accurate measurement, and for purposes of calculation, therefore, it is assumed that the entire 42 grams of diet were consumed.

The above findings are of value in interpreting the results of experiments designed to assay the amount of estrogen in various types of foodstuffs by the mouse uterine weight method. It is apparent that an increase in uterine weight does not always indicate estrogenic activity. Stob, *et al.* (5) observed

TABLE 2. EFFECT OF THE FAT CONTENT OF CHICKEN TISSUE DIETS ON THE UTERINE WEIGHT OF IMMATURE MOUSE WITH AND WITHOUT ADDED STILBESTROL (DES)

Composition of diet			DES in diet, ppb	Calc. total fat intake, gm.[a]	No. of mice	Mean initial body weight, gm.	Mean final body weight, gm.	Mean uterine weight, mgs.	Mean log ratio[b]	Change in log ratio per mcg. of DES intake[d]
Cooked lean, gm.	Rendered skin fat, gm.	Mouse diet, gm.								
275	0	27.5	0	1.3	12	9.7	10.9	6.8±0.4[c]	0.79±0.02[c]	—
270	5	27.5	0	1.8	12	9.7	11.6	6.9±0.4	0.77±0.02	—
265	10	27.5	0	2.4	12	9.8	11.8	9.6±0.8	0.85±0.03	—
275	0	27.5	6	1.3	11	9.7	11.6	42.4±3.8	1.54±0.04	3.0
270	5	27.5	6	1.8	12	9.7	12.1	29.7±3.2	1.36±0.05	2.3
265	10	27.5	6	2.4	12	10.2	12.6	21.2±1.7	1.21±0.03	1.4

[a] = Based on a total food intake per mouse of 42 grams and a determination of ether-extractable solids: Per cent fat found for the three diet compositions were 3.12, 4.39, and 5.70, respectively.
[b] = Ratio = (Uterine Weight in mg./Final Body Weight in gm.) ×10.
[c] = Standard error of the mean.
[d] = Based on a total food intake per mouse of 42 grams, a concentration of 6 ppb gives a total stilbestrol intake of 0.25 μg./mouse.

that the uterine weight of mice fed a meatmash mixture was different from those fed a straight grain-mash ration. Increasing the protein content of the straight grain mash to the approximate protein content of the meatmash mixture by the addition of casein did not alter the uterine response. Before conclusions are made that an increase in uterine weight of the mouse is due to estrogenic activity, the results should be confirmed by a microscopic examination of the morphological changes occurring in the endometrium (2, 6).

In the bioassay method used for the estimation of estrogenic residues in edible tissues (4), tissues from control or nontreated animals are always compared with tissues from stilbestrol-treated animals. Since the tissue from stilbestrol-treated chickens usually contains more fat than tissue from nontreated chickens, this factor must be taken into account in interpreting the assays. The tissues from stilbestrol-fed steers usually do not differ in composition from those of control nontreated steers.

The present experiments do not furnish an explanation for the decreased uterine weight response to stilbestrol in the presence of an increased fat content of the diet.

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DESIGN OF A BIOLOGICAL ASSAY METHOD FOR THE DETECTION AND ESTIMATION OF ESTROGENIC RESIDUES IN THE EDIBLE TISSUES OF DOMESTIC ANIMALS TREATED WITH ESTROGENS

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ABSTRACT

Experimental designs are described for the biological assay of the edible tissues of stilbestrol-treated animals for residual estrogenic activity. When residual estrogenic activity is found, an estimate of the amount in terms of stilbestrol equivalents can be made by serial dilution of the unknown or treated tissue with the control or nontreated tissue and by comparing the response of these diets with a standard dosage-response line simultaneously established by adding known amounts of stilbestrol to the control tissue diet. When residual estrogenic activity cannot be detected, the design of the assay provides a method for calculating the maximum amount which could be present and remain undetected.

The immature rat compares favorably with the immature mouse for the determination of stilbestrol in tissues. Stilbestrol was found to be from 10 to 20 times as active as the natural estrogens, estradiol-17β and estrone, in the mouse uterine weight method of assay when administered orally in the diet.

THE use of synthetic estrogens, particularly stilbestrol, for the purpose of stimulating growth and fattening of domestic farm animals used for human food has become widespread (1, 2, 3).

Early attemps to detect and measure residual estrogens in the edible tissues of treated animals by both chemical and biological methods have been reviewed by Lorenz (1). Many of these methods depended upon relatively insensitive chemical methods. Some of the studies using more sensitive bioassay methods indicated the presence of significant amounts of estrogenic activity but many of these studies were not adequately controlled.

This laboratory has undertaken the reinvestigation of the problem in order to establish independently whether or not added estrogenic residues are present in the edible tissues of beef and chickens fattened with stilbestrol and other estrogens. The present communication deals with the design of the bioassay methods used.

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The bioassay methods to be described are based on the work of Stob *et al.* (3), Turner (4), and Preston *et al.* (5). A preliminary report has been presented (6). The incorporation of the tissues into the diet of the mice eliminated the possibility of incomplete extraction of the estrogens from the tissues by solvents.

MATERIALS AND METHODS

Animals: Immature female mice (21 days old) of the White Swiss General Purpose strain were obtained from the National Institutes of Health, Bethesda, Maryland, at weaning. In a few experiments immature female rats (20–22 days old) were obtained from our own colony. These rats were originally of the Osborne-Mendel strain.

Procedure: After weaning, the animals were given ground laboratory ration (Purina chow) overnight. The next day the animals were placed in individual wire mesh cages and given the experimental diets. The animals were distributed among the treatment groups in such a way that the total initial body weight of each group was equal. This was accomplished by weighing each animal to the nearest gram and combining the animals of equal body weight. An animal was then selected at random from the lowest weight group and placed in each treatment group until all animals were distributed.

The diets were fed in glass sponge-cups which were washed daily. Frequent washing and steam sterilization of the cages was found necessary in order to avoid contamination with small amounts of stilbestrol. The diets were fed for seven days. On the eighth day of the experiment, the animals were sacrificed by cervical dislocation. Their uteri were removed and weighed, after pressing out the luminal fluid on moist filter paper.

Diets: Control tissue diets were prepared from tissues obtained from chickens and steers which did not receive stilbestrol and treated tissue diets were prepared from tissues from similar animals treated with stilbestrol. The tissues, stored in the frozen state, were thawed and then ground three times with laboratory diet (Purina chow) in the proportion of 100 parts of fresh tissue to 10 parts of laboratory diet. When estrogens were added to the tissue diets, this was done in the form of alcoholic solutions of various concentrations so adjusted that 100 grams of diet received 1 ml. of alcoholic solution of estrogen. After stirring thoroughly with an ordinary kitchen mixer, the diets were made up into 6- or 7-gram patties, placed in sealed plastic bags, and refrozen. Each test animal received one patty per day. Generally this amount of diet was all consumed and was nutritionally sufficient to permit some growth.

Calculations: The methods described by Bliss (7) for the calculation of the potency, the standard error of the potency, and for establishing the validity of the assay were used.

The typical response of mouse uterine weight to oral estrogens is a sigmoid curve. Plotting log dose versus the uterine weight response achieves linearity over a large portion of this curve. However, in the very low dosage range used in this work, linearity is achieved when the dose is plotted versus the log of the response (i.e., uterine weight, or ratio of the uterine weight in milligrams to the final body weight in grams.) The relationship between the dose and the log of the response is linear down to zero dose.

In the two-curve method to be described below, the horizontal distance between the two curves measured by

$$M = (\bar{y}_T - \bar{y}_C)/b_c,$$

where $\bar{y}_T$ and $\bar{y}_C$ are the mean log responses of the treated and control groups respectively, gives directly the concentration of estrogenic residues in the treated tissue in parts per billion of stilbestrol, after correction for the proportion of tissue in the diet. In those cases where no residual estrogen was detectable, it was desirable to estimate the maxi-

mum amount of residual estrogen that could escape detection if any were present. For this purpose the sensitivity of the assay was calculated as

$$t_{0.05} \times S_M.$$

The value "$t_{0.05}$" was taken from the t table of Fisher and Yates (11) using the degrees of freedom in the error variance s_2, and a probability level of $P = 0.05$. The value "S_M" is the standard error of the potency M. Bliss (7) describes the method of calculating the slope ratio assay, and similar methods may be found for both types of assay in Finney (9).

In illustrating the experimental designs to be described below, we have used as the response the logarithm of the ratio of the uterine weight in milligrams to the final body weight in grams, multiplied by 10 in order to avoid negative logarithms. The uterine weight of the mouse is not strictly proportional to the final body weight, but we have found this device useful as a first approximation to avoid lengthy calculations of covariance.

RESULTS

Comparison of the uterine weight response of the rat and of the mouse to stilbestrol in laboratory diet:

Four diets were prepared by adding 0, 3, 9 and 27 parts per billion (0.000, 0.003, 0.009, and 0.027 μg. per gm., respectively) of U.S.P. Reference Standard Diethylstilbestrol to the standard laboratory small animal ration. The diets were fed *ad libitum* to 22-day old female mice and to 21 to 23-day old female rats for seven days. The mean uterine weight responses are shown in Table 1. The relative sensitivity of the mouse and rat for the oral determination of stilbestrol in diets was tested by comparing the slopes of the dosage-response lines calculated from the dose and the logarithm of the ratio of uterine weight to final body weight as the response. Preliminary plotting of the results indicated that the highest dose was obviously off the linear portion of the curve; therefore, only the 0, 3 and 9 parts per billion dosage levels were used for the calculation of regression. The lack of homogeneity of the variance of the responses as shown by Bartlett's test (9) for the uterine weight was fully corrected by transformation to the log of the ratio for the mouse and greatly improved for the

TABLE 1. UTERINE WEIGHT RESPONSE OF THE IMMATURE MOUSE AND THE IMMATURE RAT TO STILBESTROL IN STANDARD LABORATORY DIET

Dose, ppb in diet	Mouse			Rat		
	No. of mice	Mean uterine wt., mg.	Mean log ratio[a]	No. of rats	Mean uterine wt., mg.	Mean log ratio[a]
0	12	12.3 ± 0.8[b]	0.9472 ± 0.0262[b]	10	43.7 ± 1.1[b]	0.7790 ± 0.0132[b]
3	11	15.8 ± 1.5	1.0387 ± 0.0282	10	54.2 ± 2.9	0.8781 ± 0.0248
9	11	33.7 ± 2.2	1.3916 ± 0.0228	10	117.9 ± 9.8	1.2232 ± 0.0360
27	11	60.6 ± 2.2	1.6241 ± 0.0148	10	155.5 ± 9.8	1.3531 ± 0.0282

[a] = Log of the ratio of the uterine weight in mg. to the final body weight in gm. multiplied by 10.

[b] = Standard error of the mean.

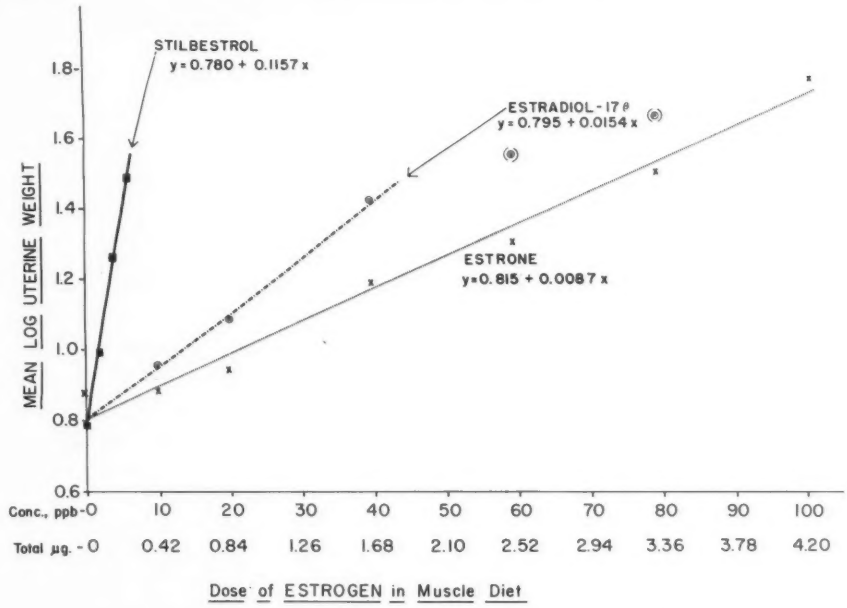

FIG. 1. Mouse uterine weight response to estrogens at low dose levels.

rat (just significant at $P = 0.05$). The slopes for both lines calculated to be $0.0505 \pm$s.e. 0.0040 indicating that the immature rat is just as sensitive to oral stilbestrol as the immature mouse. It was estimated that as little as 1 part per billion of stilbestrol in laboratory diet would give a significant response in either animal. However, the immature rat consumes 3 to 4 times as much food as the immature mouse, which is of considerable importance when only limited amounts of tissue are available.

The response of the immature rat and the ovariectomized mature mouse to oral stilbestrol has recently been compared by Wiberg and Stephenson (10). The immature rat was found most sensitive.

Relative potency of stilbestrol, estradiol-17β, and estrone in the diets of mice prepared from chicken muscle:

The relative potency of stilbestrol and the natural estrogens, when added to lean-meat diets in the low-dose range, was determined. Figure 1 shows dosage-response lines calculated by the method of least squares for stilbestrol, estradiol-17β, and estrone using the log of the uterine weight as the response. The estrogens were added to a diet of chicken lean meat containing about 9% of laboratory diet and fed at the rate of six grams per day. The cuves are linear up to about 10 parts per billion of stilbestrol, 50 parts per billion for estradiol-17β, and 100 parts per billion for estrone. The re-

sponse at 60 and 80 parts per billion was not used in calculating the regression for estradiol-17β. Since the activity of each estrogen is proportional to the slope of its regression line, we find that stilbestrol is 7.5 times as active as estradiol-17β and 13.5 times as active as estrone in this particular assay.

Experimental designs for estimating residual estrogenic activity in the edible tissues of stilbestrol-treated animals:

In all of the experimental designs to be described below, tissues from stilbestrol-treated animals are compared with tissues from control animals in each assay. The assumption is made that if naturally occurring estrogens are normally present in the tissues, their amounts will be comparable and any difference between the uterine weight response of mice fed control and treated tissue diets will be due to residual estrogen in the treated tissue. In order to quantitate the amount of estrogen found and to provide a measure of the sensitivity of the assay, known increments of stilbestrol were added to control tissue diets to provide a standard curve for each assay. The inclusion of standard curve with each assay also corrects for day-to-day variation in the urterine weight response of the mouse.

We have noted along with others (3, 4, 6, 10) that the weight of the immature mouse or rat uterus depends upon the type of diet (or tissue) fed the experimental animal. While we are not convinced that the apparent uterine-stimulating activity of some diets as compared with others is in every case due to estrogenic substances, this is another reason for including the same type of control tissue in every assay for residue estrogen in treated tissue.

Design A. Two-curve method.

In this design, stilbestrol was added to both control and treated tissue diets fed the mice at concentrations of 0, 2, 4 and 6 parts per billion. Two curves were obtained, one for the treated tissue, which serves as the "unknown" curve, and one for the control tissue, which serves as the "standard" curve. Figure 2 shows the results of a typical assay by this method. The upper two curves show the results when the dose in parts per billion is plotted versus the response measured as the mean uterine weight. Since there was no significant difference in the final body weights of the mice between the various groups, the same general curves would have been obtained if the response had been plotted as the ratio of the uterine weight in milligrams to the final body weight in grams. Definite curvature is indicated which is eliminated by plotting the dose versus the log of the ratio as shown by the lower two curves. The latter curves are linear and parallel and differ significantly from each other ($P < 0.05$). Any displacement of the treated curve upward along the response axis from the control curve indicates residual estrogenic activity in the treated tissue, and the amount of displacement measures the amount of residual estrogenic activity in equi-

valents of stilbestrol. The amount of residual estrogenic activity in this case calculated to be 0.77 ±s.e. 0.38 part per billion. The sensitivity calculates to be 0.76 part per billion.

When the concentration of residual estrogenic activity in the treated tissue was greater than two parts per billion, the curves were no longer parallel since the response was then on a higher portion of the dose-response curve. Figure 3 shows curves plotted from data of a typical assay of this sort. The assay is invalid and a calculation of the amount of estrogen present cannnot be made.

Design B. The range finder.

In order to determine the approximate level of estrogenic activity in a tissue suspected of containing more than two parts per billion, a second experimental design termed the "range finder" was used. This consisted of feeding control tissue diets to which 0, 3, and 6 parts per billion of stilbestrol had been added to three groups of mice and at the same time feed-

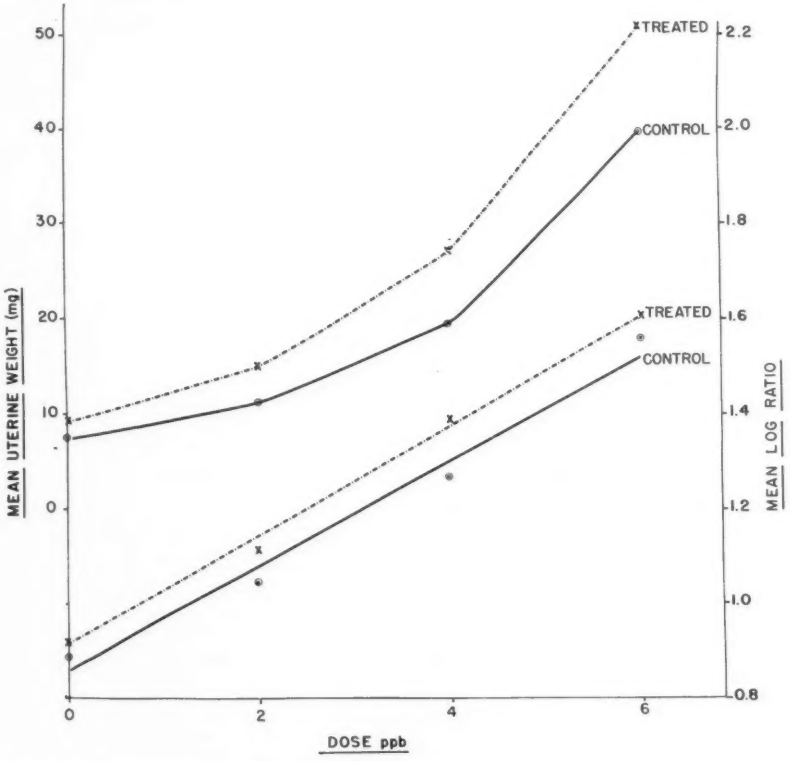

FIG. 2. Results of a typical assay. Upper curves, dose versus response; lower curves, dose versus log response.

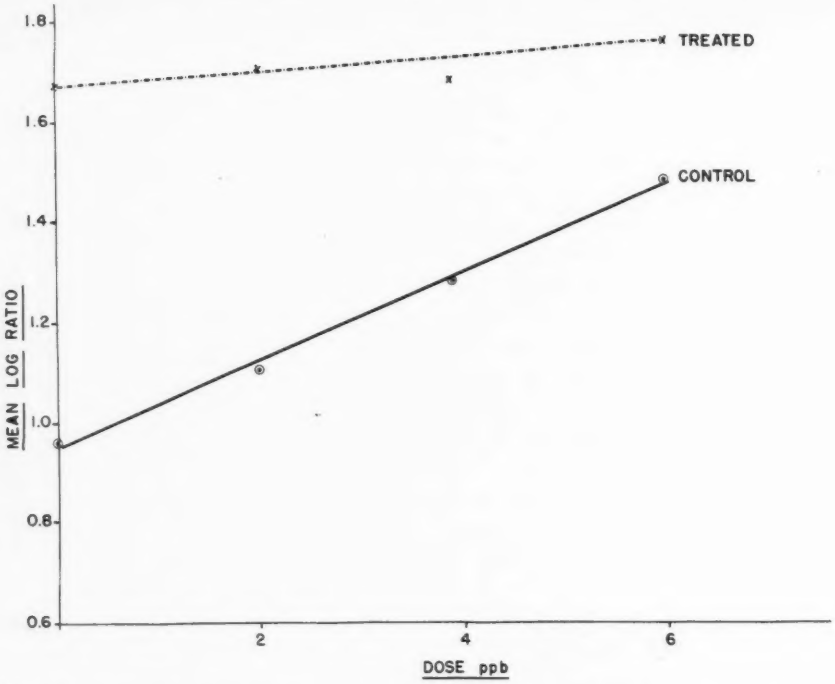

FIG. 3. Results of a typical assay where response indicates a concentration of residual estrogen greater than 2 ppb stilbestrol equivalents.

ing tissue diets diluted with 0, 50, 75, 87.5 and 93.75 per cent of control tissue diet to five other groups of mice. The design and results of a typical range-finder experiment are illustrated in Table 2 and Figure 4. The plotted values indicate that the treated tissue contains residual estrogenic activity equivalent to about 6 parts per billion of stilbestrol. A satisfactory concentration of treated diet in a mixture of treated and control diet for a confirmatory assay would be 25 per cent.

Since the curves are linear and pass through a common origin, the data can be calculated directly as a balanced slope-ratio assay. The data from Groups A, B, C, G, and H were used in this manner for calculation of the amount of residual estrogenic activity in the treated tissue as described by Bliss (7). The amount found was 6.9 $\pm$ s.e. 0.5 parts per billion in stilbestrol equivalents.

Design C. Confirmatory dilution assay.

In this design the treated tissue diet was diluted with control tissue diet on the basis of the results of the range-finder experiment so that the expected concentration of residual estrogenic activity in stilbestrol equiva-

TABLE 2. DESIGN AND RESULTS OF A TYPICAL RANGE FINDER EXPERIMENT

Group	Number of animals	Proportional parts of		Added stilbestrol in ppb	Mean final body wt.	Mean uterine wt.	Mean log ratio[a]
		Control diet	Treated diet				
A	6	100	0	0	12	7.5	0.80
B	6	100	0	3	12	27.4	1.29
C	6	100	0	6	12	47.4	1.60
D	6	93.75	6.25	0	11	6.6	0.86
E	6	87.5	12.5	0	11	10.3	0.98
F	5	75	25	0	11	14.6	1.09
G	6	50	50	0	12	19.9	1.21
H	6	0	100	0	13	61.0	1.68

[a] = Log of the ratio of the uterine weight in mg. to the final body weight in gm. multiplied by 10.

lents was between 1 and 2 parts per billion. Stilbestrol at concentrations of 0, 2, 4, and 6 parts per billion was then added to the control tissue diet and to the diluted treated tissue diet and the assay performed as in Design A.

Design D. Single point assays.

In those cases where there was insufficient tissue to perform a full assay

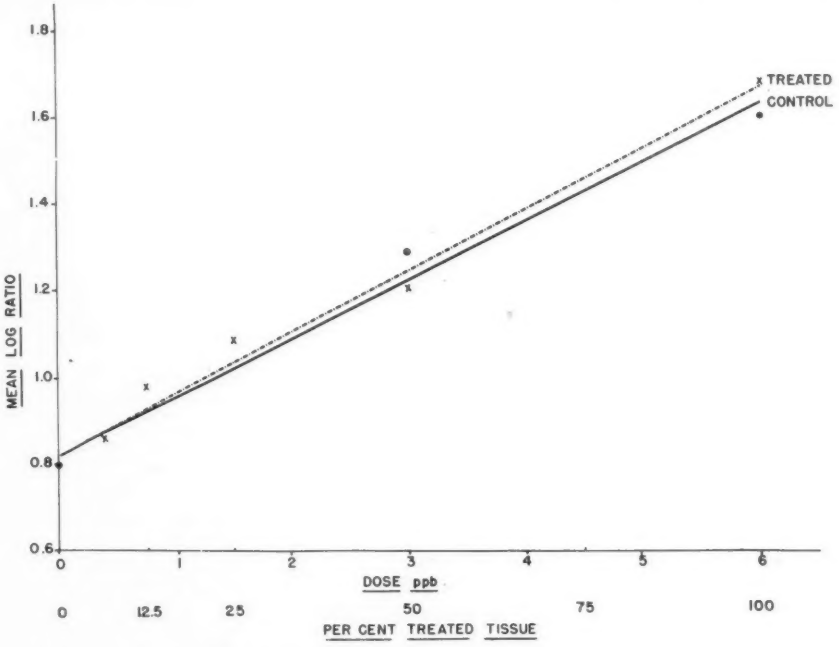

FIG. 4. Results of a typical range finder experiment. Solid line and circles show response of stilbestrol in control tissue diet. Broken line and crosses show responses of treated tissue diet diluted with various percentages of control tissue diet.

by the two-curve method, single point assays were performed by feeding control tissue diet without spiking to one group of animals and similarly the treated tissue diet to another group of animals. A statistically significant difference between the mean uterine weights of the two groups of mice may indicate residual estrogenic activity. Attempts to estimate quantitatively the amount of estrogen present by comparing the responses with standard curves established independently in another tissue or in laboratory diet are untenable. The results should be confirmed in another experiment in which sufficient tissue for a full assay is obtained.

DISCUSSION

Stob *et al.* (3), and Preston *et al.* (5), dried the tissue to be assayed before incorporating it into diets for the mice. We have followed the method of Turner (4) where the tissue is fed without drying. The response of the uterine weight depends upon the total amount of estrogen consumed. Immature mice consume about six grams of fresh tissue diet but only a little over two grams of the laboratory mouse diet or of the dried tissue diet, depending upon the water content. Therefore, in order to obtain an equal uterine weight response, one must add about three times as much stilbestrol to the laboratory mouse diet as is necessary for the fresh tissue diet. It would appear, therefore, that no significant increase in sensitivity of the assay is gained by drying the tissue.

The finding that stilbestrol is about ten to twenty times as active as the natural estrogens in this assay method was to be expected from the work of Sondern and Sealey (8) who showed that stilbestrol was twenty times as active as estradiol-17β and twenty-five times as active as estrone when administered orally by stomach tube to castrate adult mice using the vaginal smear as the response. Using the rat as the test animal, these authors found that stilbestrol was sixty-five and eighty times as active as estradiol-17β and estrone respectively. When assayed on spayed adult mice and rats by subcuaneous injection, stilbestrol was found to have about the same order of potency as estrone, i.e., one to two times as potent as estrone and one-fifth to one times as active as estradiol-17β. From the above observations, it would appear that the present method should be more specific for the assay of stilbestrol in the edible tissues of stilbestrol-treated animals since relatively large amounts of the naturally occurring estrogens would have to be present in order to elicit a response.

Acknowledgment

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PROGESTATIONAL ACTIVITY OF CERTAIN 19-NOR STEROIDS AND PROGRESTERONE DERIVATIVES[1]

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ABSTRACT

Seven 19-norsteroids, 17α-ethinyl-19-nortestosterone (I), 17α-ethinyl-5(10)-estraenolone (II), 17α-ethyl-19-nortestosterone (III), 17α-methyl-19-nortestosterone (IV), 17α-propyl-19-nortestosterone (V), 17α-butenyl-19-nortestosterone (VI), and 17α-allyl-19-nortestosterone (VII), and two progesterone derivatives—17α-hydroxyprogesterone-17-acetate (VIII) and 17α-hydroxy-6α-methyl-progesterone acetate (IX) have been administered subcutaneously or orally into Clauberg rabbits to demonstrate their progestational activity by estimation of endometrial carbonic anhydrase content and measurement of the uterine G/M ratio.

The increase of endometrial carbonic anhydrase content induced by treatment with each compound correlates well with that of the G/M ratio which measures uterine proliferation. Similarly, the relative potencies by the enzyme test correspond to those obtained by the G/M assay either in subcutaneous treatment or in oral administration.

The relationship between the progestational activity and the length of 17-side-chain in the 17-substituted-19-nortestosterones found by Saunders *et al.* (3) seems to be applicable to the potencies obtained by subcutaneous injection but not to those found on oral administration. Among the 17-substituted-19-nortestosterones, the allyl-compound is the most active in the subcutaneous assay, but the most active by oral route is the ethyl-compound.

Three 19-norsteroids, I, II and IV are much more active by the oral route than by the subcutaneous route, while the others are quite opposite. The progestational activity of progesterone administered either parenterally or orally is increased by 17α-acetoxylation, and further increase is obtained by 6α-methylation of this acetate ester.

A NUMBER of 19-norsteroids and progesterone derivatives have been demonstrated as progestational substances in animals and in human beings (1, 2, 3, 4, 5, 6, 7, 8). Most of the relative potencies of those compounds have been estimated only by the minimum effective dose which produced a detectable change in the histological condition of uterine endometrium.

In our previous report, it was demonstrated that the content of carbonic anhydrase in the endometrium of the Clauberg rabbit is closely correlated with the measurement of pseudo-pregnant proliferation (glandular/total muscosal area—G/M ratio) both in the accuracy of the dose-response re-

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gression of progesterone and in a comparative assay with Δ^4-pregnene-3-ol-20-one, suggesting the usefulness of the carbonic anhydrase method as a standard assay procedure (9). Subsequently, in our laboratory, the progestational activity of various compounds has been tested quantitatively in this manner.

In this paper we present: (a) the dose-response for ten steroids administered subcutaneously or orally to the conventional Clauberg rabbit, (b) the comparison of the dose-response curves in the enzyme test with those in the G/M test, (c) the comparative potency ratios of the compounds in both types of assay, and (d) the oral/subcutaneous ratio of each compound.

The steroids used in this experiment are as follows: progesterone (P), 17α-ethinyl-19-nortestosterone (I), 17α-ethinyl-5(10)-estraen-17β-ol-3-one (II), 17α-ethyl-19-nortestosterone (III), 17α-methyl-19-nortestosterone (IV), 17α-propyl-19-nortestosterone (V), 17α-butenyl-19-nortestosterone (VI), 17α-allyl-19-nortestosterone (VII), 17α-hydroxyprogesterone-17-acetate (VIII) and 17α-hydroxy-6α-methyl-progesterone-17-acetate (IX).

METHODS

Following the Clauberg method (10), immature rabbits weighing approximately 1.5 kg. are injected subcutaneously once daily with 5 μg. of estradiol for 6 days. After this period of estrogen-priming, the rabbits are injected subcutaneously or force fed by gavage once daily with various doses of progesterone or other steroids. The animals are sacrificed 24 hours after the last injection or feeding. All compounds are dissolved in Sesame Oil U.S.P.

The procedure of extraction of endometrial carbonic anhydrase and the measurement of uterine G/M ratio used as an indicator of progestational proliferation are the same as those described in the previous paper (9). The enzyme activity is determined by the colorimetric method of Philpot and Philpot (11), but, the volume of the reaction medium is reduced to one-half of the original, so that a lesser amount of enzyme extract is required, and more sensitivity can be obtained in the estimation.

The modified apparatus for the enzyme estimation is illustrated in Figure 1. The procedure for enzyme determination is as follows:

(A) Reagents:

1. Solution A_1—solution saturated with Na_2CO_3 at 5° C.
2. Solution A_2—27.3 vol. per cent of Solution A_1.
3. Solution B—$NaHCO_3$ 0.221 grams in 1000 ml. distilled water.
4. BTB—Brom Thymol Blue, 0.1 per cent aqueous.

(B) Procedure:

1. Half-fill 500 ml. aspirator bottle with Solution B.
2. Open the CO_2 tank and start the bubbling in order to saturate Solution B with CO_2 (about 1 bubble/second for 10–15 minutes).
3. Put 0.1 per cent BTB in the burette (5 ml.).
4. Put 0.1 ml. BTB in each test tube (1.5 cm.×15 cm.).
5. Fill burette (50 ml.) with Solution B saturated with CO_2.
6. Put 4 or 5 ml. of saturated Solution B into each test tube.
7. Put a stopper on each test tube immediately after step 6.
8. All test tubes must be kept in an ice-bath.

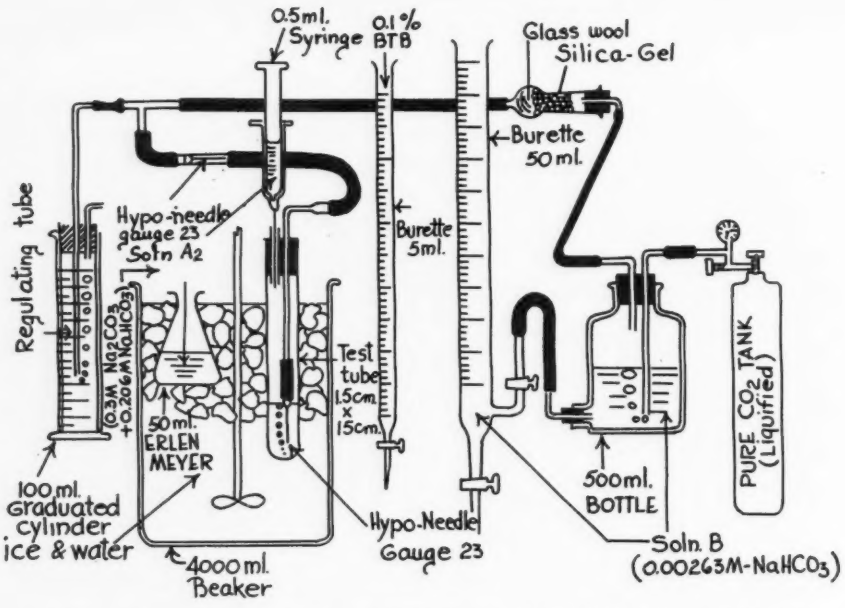

FIG. 1. Determination of Carbonic Anhydrase Activity. (Miyake's modification of Philpot & Philpot)

9. Fill 100 ml. graduated cylinder with 100 ml. water. Adjust regulating tube at approximately 35 ml. level.
10. Connect all tubing as shown in Figure 1.
11. Make ice-bath in 4000 ml. beaker and start stirrer.
12. Put Solution A_2 in 50 ml. Erlenmeyer and put in ice-bath.
13. Start the control measurement.
    a. Add distilled water to test tube to make a volume of 5.6 ml.
    b. Add one drop of octyl alcohol.
    c. Put test tube in ice-bath and bubble CO_2 through for 2 minutes to make equilibrium.
    d. Add 0.5 ml. Solution A_2 to test tube and at the same time start the stop watch.
    e. When color turns green-yellow from initial blue, record the time.
    f. Repeat, adjusting the regulator until the reaction time is 65–75 seconds. This is the control reaction time.
14. Start the measurement of enzyme activity.
    a. Substitute the enzyme extract for distilled water and proceed as in Step 13, a. through e.
    b. Adjust the dilution of enzyme extract until the reaction time is 25–30 seconds (one enzyme unit—1 EU).
15. The enzyme unit per gram wet tissue (EU/g.) is calculated as follows:

$$EU/g = \frac{1000}{C \cdot V} \cdot \frac{1}{2},$$

C: Concentration of extract (mg./ml.)

V: Volume of extract which is required to shorten the reaction time from 65–75 to 25–30 seconds (ml./EU).

Because the volume of the reaction medium is reduced to one-half of the original of Philpot and Philpot, $\frac{1}{2}$ is multiplied in the calculation in order to make it

TABLE 1. THE EFFECT OF CERTAIN 19-NORSTEROIDS AND PROGESTERONE DERIVATIVES ON THE ENDOMETRIAL CARBONIC ANHYDRASE ACTIVITY AND THE G/M RATIO OF CLAUBERG RABBITS' UTERI

(A) Subcutaneous injection

Compound	Total dose (mg./rabbit)	No. of rabbits	EU/g. wet tissue, mean ± S.E.	G/M ratio of uterus, mean ± S.E.
P	0.5	12	37.8 ± 3.2	0.609 ± .017
	2.0	14	102.6 ± 8.3	0.745 ± .010
I	0.5	7	18.4 ± 1.7	0.506 ± .039
	2.0	8	23.5 ± 5.2	0.523 ± .019
	8.0	5	66.1 ± 5.5	0.732 ± .057
II	2.0	9	23.3 ± 4.2	0.463 ± .020
	4.0	8	22.9 ± 2.5	0.541 ± .022
	8.0	9	26.9 ± 4.8	0.555 ± .022
III	0.125	5	27.1 ± 3.8	0.587 ± .043
	0.5	7	77.4 ± 8.0	0.692 ± .084
	2.0	8	97.5 ± 8.2	0.744 ± .019
	8.0	7	81.6 ± 6.6	0.674 ± .021
IV	0.5	7	23.4 ± 1.7	0.547 ± .015
	2.0	8	29.7 ± 4.9	0.596 ± .024
	8.0	6	56.5 ± 8.6	0.677 ± .025
V	0.125	13	23.8 ± 1.7	0.535 ± .017
	0.5	8	85.9 ± 8.5	0.721 ± .010
	2.0	7	94.2 ± 7.9	0.780 ± .008
VI	0.125	12	36.8 ± 4.7	0.542 ± .022
	0.5	9	91.2 ± 5.6	0.702 ± .017
	2.0	8	88.7 ± 2.7	0.767 ± .012
VII	0.031	7	16.9 ± 1.2	0.443 ± .012
	0.125	6	36.6 ± 4.5	0.553 ± .037
	0.5	9	135.6 ± 8.7	0.713 ± .015
	2.0	5	157.8 ± 11.0	0.763 ± .013
VIII	0.031	8	16.4 ± 0.7	0.502 ± .019
	0.125	16	61.2 ± 6.3	0.603 ± .023
	0.5	8	104.7 ± 10.1	0.718 ± .015
	2.0	7	122.2 ± 11.0	0.745 ± .020
IX	0.008	8	22.8 ± 3.2	0.522 ± .022
	0.031	8	83.7 ± 3.9	0.691 ± .016
	0.125	11	126.4 ± 6.1	0.721 ± .016
	2.0	7	120.3 ± 7.7	0.735 ± .014

P: Progesterone
I: 17α-ethinyl-19-nortestosterone
II: 17α-ethinyl-5(10)-estraen-17β-ol-3-one
III: 17α-ethyl-19-nortestosterone
IV: 17α-methyl-19-nortestosterone
V: 17α-propyl-19-nortestosterone
VI: 17α-butenyl-19-nortestosterone
VII: 17α-allyl-19-nortestosterone
VIII: 17α-hydroxyprogesterone-17-acetate
IX: 17α-hydroxy-6α-methyl-progesterone-17-acetate

equivalent to the definition of the enzyme unit of Keilin and Mann (12), and to make it possible to compare the data in this paper with those in the previous paper (9).

RESULTS

In Table 1, we present the mean data on endometrial carbonic anhydrase activity and glandular/total mucosal area (G/M) in a series of animals in-

TABLE 2. THE EFFECT OF CERTAIN 19-NORSTEROIDS AND PROGESTERONE DERIVATIVES ON THE ENDOMETRIAL CARBONIC ANHYDRASE ACTIVITY AND THE G/M RATIO OF CLAUBERG RABBITS' UTERI

(B) Oral administration

Compound	Total dose (mg./rabbit)	No. of rabbits	EU/g. wet tissue, mean ±S.E.	G/M ratio of uterus, mean ±S.E.
P	2.0	6	12.9± 0.7	0.328±0.011
	8.0	4	13.1± 3.0	0.364±0.012
I	0.5	6	34.9± 7.3	0.481±0.047
	2.0	5	73.2± 9.2	0.561±0.016
	8.0	4	85.6± 9.8	0.682±0.021
II	2.0	5	48.6± 4.4	0.574±0.024
	8.0	4	70.8±10.3	0.660±0.037
III	0.125	5	42.3± 9.8	0.476±0.035
	0.5	6	71.4±11.2	0.683±0.041
	2.0	5	116.2±17.4	0.709±0.033
	8.0	5	107.5±10.2	0.757±0.045
IV	0.5	5	40.7± 5.8	0.577±0.013
	2.0	6	100.2±17.4	0.657±0.020
	8.0	3	90.5±21.6	0.684±0.024
V	0.5	7	42.7±12.8	0.521±0.053
	2.0	6	110.8±22.6	0.643±0.021
	8.0	5	121.1±28.7	0.691±0.033
VI	0.5	6	41.2±11.7	0.572±0.023
	2.0	6	86.6±18.7	0.701±0.018
	8.0	4	84.0±21.1	0.673±0.006
VII	0.5	5	36.1± 5.9	0.527±0.025
	2.0	5	96.7±12.3	0.619±0.050
	8.0	4	125.1±16.5	0.642±0.020
VIII	0.5	7	31.5± 3.5	0.514±0.033
	2.0	9	79.1±12.3	0.624±0.024
	8.0	10	138.9±28.5	0.681±0.013
IX	0.031	6	25.5± 5.9	0.481±0.041
	0.125	5	71.1±15.1	0.619±0.059
	0.5	4	161.6± 4.4	0.707±0.010
	2.0	6	130.2± 8.4	0.739±0.030

P: Progesterone
I: 17α-ethinyl-19-nortestosterone
II: 17α-ethinyl-5(10)-estraen-17β-ol-3-one
III: 17α-ethyl-19-nortestosterone
IV: 17α-methyl-19-nortestosterone
V: 17α-propyl-19-nortestosterone
VI: 17α-butenyl-19-nortestosterone
VII: 17α-allyl-19-nortestosterone
VIII: 17α-hydroxyprogesterone-17-acetate
IX: 17α-hydroxy-6α-methyl-progesterone-17-acetate

TABLE 3. RELATIVE PROGESTATIONAL POTENCY OF 19-NORSTEROIDS AND PROGESTERONE DERIVATIVES

Compound	Relative progestational potency			
	Subcutaneous injection		Oral administration	
	enzyme test	G/M test	Enzyme test	G/M test
Progesterone	1.0*	1.0*	—	—
17-Ethinyl-19-nortestosterone	0.1	0.2	1.0	0.5
17-Ethinyl-5(10)-estraenolone	—	—	0.3	0.5
17-Ethyl-19-nortestosterone	2.6	2.6	3.2	4.4
17-Methyl-19-nortestosterone	0.1	0.1	1.5	2.0
17-Propyl-19-nortestosterone	2.9	2.6	1.7	1.2
17-Butenyl-19-nortestosterone	3.5	2.4	1.3	2.2
17-Allyl-19-nortestosterone	5.8	2.7	1.3	1.1
17-Hydroxyprogesterone acetate	7.3	3.3	1.0†	1.0†
17-Hydroxy-6-methyl-progesterone-acetate	44.1	33.8	13.6	13.0

* Standard in subcutaneous injection.
† Standard in oral administration.
— Activity is too low to estimate.

jected subcutaneously with progesterone and other steroids. In both types of measurement, enzyme assay and G/M assay, compounds I, II and IV are much less active than progesterone, and the other 19-norsteroids (III, V, VI and VII) and two progesterone derivatives (VIII and IX) are much more active. The maximum enzyme responses induced by all these 19-norsteroids, except VII, are lower than those of progesterone and its derivatives. The allyl-compound (VII) shows a steeper S-shaped dose-response curve which has the highest maximum response and which can be distinguished from the others. In the G/M measurement, however, there are no significant differences between the maximum responses of the various compounds. Excluding this point, the shapes of the dose-response curves obtained by the enzyme assay closely resemble those obtained by the G/M assay.

In Table 2, we present the data obtained following the oral administration of these steroids. The maximum responses in the enzyme assay of 17-substituted 19-nortestosterones, except for the allyl-compound, appear to be lower than those of progesterone derivatives in the oral administration as well as in the subcutaneous treatment. But there are no such significant differences between their maximum responses in the G/M measurement. When administered orally, progesterone is completely inactive at doses up to 8 mg. per animal, while the other compounds are all active and give obvious dose-response curves, most of which are shifted to a higher dosage level when compared with those in the subcutaneous assay.

In Table 3, we present the comparative potency ratios of 19-norsteroids and progesterone derivatives calculated according to a four-point assay procedure in the range of dosage in which their dose-response curves are

TABLE 4. RELATIVE PROGESTATIONAL POTENCY OF 19-NORSTEROIDS AND PROGESTERONE DERIVATIVES ADMINISTERED ORALLY AGAINST THE SAME COMPOUNDS INJECTED SUBCUTANEOUSLY

(The compounds injected subcutaneously = 1)

Compound	Oral/Subcutaneous ratio	
	enzyme test	G/M test
Progesterone	0	0
17-Ethinyl-19-nortestosterone	5.5	1.4
17-Ethinyl-5(10)-estraenolone	—	5.4
17-Ethyl-19-nortestosterone	0.8	0.6
17-Methyl-19-nortestosterone	9.6	2.9
17-Propyl-19-nortestosterone	0.4	0.2
17-Butenyl-19-nortestosterone	0.2	0.3
17-Allyl-19-nortestosterone	0.2	0.1
17-Hydroxyprogesterone acetate	0.1	0.1
17-Hydroxy-6-methyl-progesterone acetate	0.2	0.2

parallel with that of the compound used as the standard. Progesterone and 17-hydroxyprogesterone acetate are used as standards in the subcutaneous and the oral assays respectively. The data demonstrate that the relative potencies by the enzyme test correspond roughly to those obtained with the G/M ratio either in the subcutaneous or in the oral assay. In order of rank, however, the activities by the subcutaneous route are quite different from those by the oral route. The order of activity by subcutaneous route are IX > VIII > VII > VI ≒ V ≒ III > P > I ≒ IV > II, while by the oral route IX > III > IV ≒ V ≒ VI ≒ VII > I ≒ VIII > II ≫ P.

In Table 4, we present the oral/subcutaneous ratios of these compounds obtained by calculating the potency of each compound administered orally against the same compound injected subcutaneously. The data demonstrate that the compounds I, II and IV are much more active orally than subcutaneously, while with the other compounds the reverse is true.

DISCUSSION

In this paper, the correlation between the content of endometrial carbonic anhydrase and the degree of uterine proliferation (G/M ratio) is quantitatively demonstrated not only for progesterone but for the other nine progestational substances, indicating the specificity of the enzyme test. This agrees with the findings of Lutwak-Mann (4, 13).

Comparing the dose-reponse curves of the various compounds, there are some differences between the magnitude of their maximum responses in the enzyme test. In the G/M assay, however, no significant differences between their maximum responses are observed, because any dosage which is able to cause over an 80 EU/g. response in the enzyme test gives an almost maximum response in the G/M assay. It is, therefore, advisable to use the endometrial carbonic anhydrase measurement together with the histo-

logical examination when the progestational activity is discussed as a dose-response curve.

Judging the relative progestational potency of the various compounds injected subcutaneously with their dose-response curves, the three 19-norsteroids, 17-ethinyl-(I), 17-methyl-19-nortestosterone (IV) and 17-ethinyl-5(10)-estraenolone (II) are much less active than progesterone; I and IV have approximately 1/10 the activity of progesterone and II is too low to estimate its relative potency. These results do not agree with the previous data (2) which were estimated on the basis of minimum effective dose. When administered orally, these three compounds I, II and IV are much more active, the oral/subcutaneous ratios being remarkably high, while oral progesterone is inactive at total doses up to 8 mg. per animal. These findings suggest that these three 19-norsteroids may be metabolized into more active compounds in the liver and/or in the gut.

In the subcutaneous assay, the relative potencies of 17α-substituted 19-nortestosterones, methyl, ethinyl, ethyl, propyl, allyl and butenyl compound are 0.1, 0.1, 2.6, 2.9, 5.8 and 3.5 respectively in the enzyme test, and 0.1, 0.2, 2.6, 2.6, 2.7 and 2.4 in the G/M assay. These results roughly correspond to the findings of Saunders *et al.* (3) who noticed that the progestational activity of 19-nortestosterone increases as the 17α-side-chain is lengthed. In the oral assay, however, the above relationship between activity and 17-side-chain is not observed; methyl, ethinyl, propyl and allyl compound having about the same potency, and the ethyl compound being approximately 2–3 times as active as the others.

17α-Hydroxyprogesterone acetate is not only more active than progestrone in the subcutaneous test, but also effective orally, as are the series of 17-substituted-19-nortestosterones. This agrees with the finding of Davis and Wied (8) who demonstrated that 17-hydroxyprogesterone acetate is an orally-effective progestational substance in women.

17α-Hydroxy-6α-methyl-progesterone-17-acetate is the most active progestational substance either parenterally or orally among the steroids presented in this paper. It has approximately 30–40 times the activity of progesterone in the subcutaneous assay, and about 13 times the activity of 17-hydroxyprogesterone acetate in the oral assay.

It is, therefore, demonstrated here that the progestational activity of progesterone is increased by 17α-acetoxylation, and is further increased with that along with 6α-methylation. It is notable that the glucocorticoid activity of cortisone is also increased by 6α-methylation.

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EFFECT OF VARIOUS ADRENAL STEROIDS ON PLASMA MAGNESIUM AND THE ELECTROCARDIOGRAM OF ADRENALECTOMIZED DOGS[1]

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ABSTRACT

Plasma Mg increases in adrenal insufficiency and remains elevated for several days after plasma K and other electrolytes have returned to normal and symptoms have disappeared under medication with adrenal steroids such as 2-methyl-9α-fluorohydrocortisone, aldosterone and desoxycorticosterone. Electrocardiographic changes commonly occur in adrenal insufficiency and cardiac arrythymia may be severe enough to cause collapse and death. These disturbances are generally assumed to be due to hyperkalemia *per se* but other factors such as low plasma Na, Cl and plasma volume plus high plasma Mg may be involved along with increased plasma K in inducing the cardiac irregularities. The effect of the various steroids upon water and electrolyte shifts are also discussed.

THE plasma Mg is known to increase in adrenal insufficiency (1, 2). Although most of the observations were made on plasma, Conway and Hingerty (3) state that muscle cells also participate in the increase. It has been posutlated (4) that the increased Mg levels encountered may be largely responsible, through inhibition of certain enzyme activities, for most of the metabolic disturbances characteristic of adrenal insufficiency. It has also been reported that Mg and Na retention and increased excretion of K are common in post-operative surgical cases suggesting that renal elimination of Mg may be influenced by the adrenal cortex. Mg is also retained in these patients following ACTH therapy. Thus in individuals subjected to major surgery, Mg is said to follow closely the behavior of Na (5). However, to anticipate presentation of the results of the following experiments, the situation is apparently quite different with regard to behavior of these ions in adrenalectomized dogs during insufficiency and recovery on steroid treatment since plasma Mg definitely tends to parallel the behavior of K rather than Na.

The present study is concerned with plasma Mg, K and Na of fasted adrenalectomized dogs receiving various types of steroid hormones. Since

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[2] New Jersey Heart Association.

plasma Mg and K are substantially higher than normal in dogs exhibiting insufficiency and undergo sharp reduction in response to adrenal steroid treatment, electrocardiographic studies were also made.

MATERIALS AND METHODS

Adult, male dogs were used; their adrenals had been removed 6 months to one year previously. When not in use the animals were maintained in normal health by daily injections of DCA plus a supplement of 1 gm. NaCl/day in the food. After discontinuing hormone the salt supplement was withheld and the dogs were fed a daily ration containing 1.47 gm. of Na and 0.94 gm. of K. Blood samples were taken and the animals permitted to develop adrenal insufficiency. When severe symptoms appeared, samples were again drawn, the bladder drained and the various steroids injected i.v. The dogs were then placed in a metabolism cage without food or water for 48–72 hours or until all symptoms of adrenal insufficiency disappeared and the plasma Na and K had returned to their initial values.

Plasma Na and K were determined by flame photometry; Mg and Ca were analyzed spectrophotometrically according to the method of Zak *et al.* (6). The determinations were performed on a Bausch and Lomb "Spectronic 20" making it necessary to reduce the quantity of dyes recommended by Zak in order to facilitate readings on the B and L apparatus. The steroids were solubilized for i.v. injection in 25% alcohol, 25% propylene glycol and 50% water. They were first dissolved in hot absolute ethanol, then hot propylene glycol added dropwise followed by hot water added very slowly with constant stirring.

RESULTS

Effect of 2-methyl-9 α-fluorohydrocortisone (2-methyl FF) on plasma Mg and other electrolytes of fasted adrenalectomized dogs recovering from insufficiency

DCA was withheld from 8 animals; they were permitted to develop marked insufficiency symptoms then injected three times daily with small quantities of 2-methyl FF. The total dose given over the 48 hour recovery period varied from 2.5–5 mg. as needed to effect recovery. Figure 1 summarizes the essential data; each bar in the graph, with the exception of

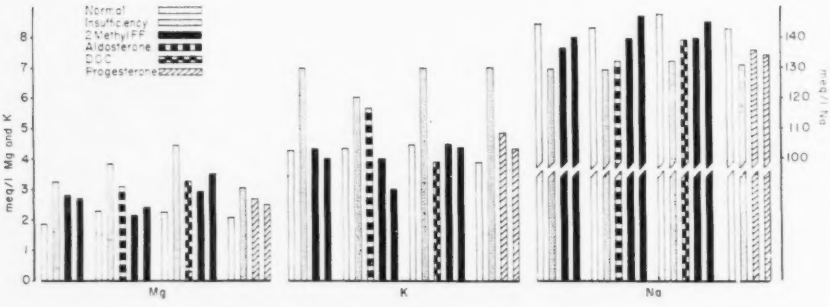

FIG. 1. Beginning with the dotted bar (point of 1st injection), each succeeding bar on the graph represents a 24 hour period of steroid treatment. All determinations were made on plasma.

those experiments where aldosterone, desoxycorticosterone and progesterone were used, represents an average of 8 animals.

The usual decline in plasma Na and sharp rise in K took place as symptoms developed but changes in Ca were negligible and will not be discussed further. Mg increased in the plasma and like K attained its greatest concentration when symptoms were severe. Following hormone treatment Mg decreased along with K but did not decline to the control level until 5–7 days after the K had fallen to its initial value (Fig. 1).

Changes in the electrocardiogram (ECG), of these animals during insufficiency were essentially similar to those recorded in the literature as characteristic of adrenalectomized dogs with elevated plasma K (7, 8, 9, 10). However, following 2-methyl FF therapy and disappearance of symptoms with return of activity and vigor, resumption of the normal ECG pattern during the repolarization stage was much delayed. Thus return of plasma K to its preinsufficiency level was not accompanied by return to normal of either the ECG or plasma Mg (Fig. 2).

Plasma Mg, K and Na in fasted adrenalectomized dogs treated with aldosterone, desoxycorticosterone and progesterone while exhibiting severe insufficiency

Sufficient crystalline *d, l*-aldosterone-21-monoacetate was obtained to test the ability of this mineralocorticoid to revive 2 fasted dogs from adrenal failure. In our experience the compound proved incapable of abolishing symptoms or improving the animal's condition in the absence of food and water even though administered i.v. in larger doses than are required for 2-methyl FF to restore the dog to normal health. One dog died 6 hours after receiving 4 mg of aldosterone; the remaining animal showed marked deterioration during the 24 hours after receiving 10 mg. of the steroid in three doses of 6, 2, and 2 mg respectively. At the end of this interval of aldosterone treatment the condition of the animal was such that it became imperative to administer large doses of 2-methyl FF in order to save the dog. Activity and vigor rapidly returned and the fasting period was extended for an additional 48 hours without harm to the animal.

Plasma Mg declined slightly under the stimulus of aldosterone but fell to its control level when 2-methyl FF was later injected. Apparently the combined treatment of mineralocorticoid plus glucocorticoid was more effective in reducing plasma Mg, presumably by increasing renal elimination, than either type of steroid given singly. One dog was permitted to develop insufficiency with lowered arterial pressure, plasma volume, elevated plasma Mg and K and lowered Na and Cl. He was then injected i.v. with 125 mg. of DOC given in three doses of 75, 25 and 25 mg. over the ensuing 24 hours. Food and water were withheld for the following 72 hours. Treatment with this steroid was ineffective in relieving symptoms of the fasted animal. The blood pressure and plasma volume continued to decline

and the dog remained weak and spastic. The plasma Na rose sharply, Mg and K declined but the increase in plasma Na and fall in K is probably accounted for by the excretion of 605 cc. of practically sodium-free urine (3.9 M.eq/Na) but containing considerable K (21.6 M.eq). The dog exhibited serious symptoms at the end of the 24 hours of DOC treatment and after drawing blood samples was too weak to stand or walk. He was then injected with 2-methyl FF and restored to normal activity and vigor within 48 hours despite continued lack of food and water. The plasma volume, arterial pressure and plasma Na and K returned to control levels but the Mg remained elevated.

DOC, even in these massive doses failed to induce an internal shift of fluid and electrolyte but did cause renal retention of Na and excretion of K. No improvement in the animal's condition was evident until 2-methyl FF

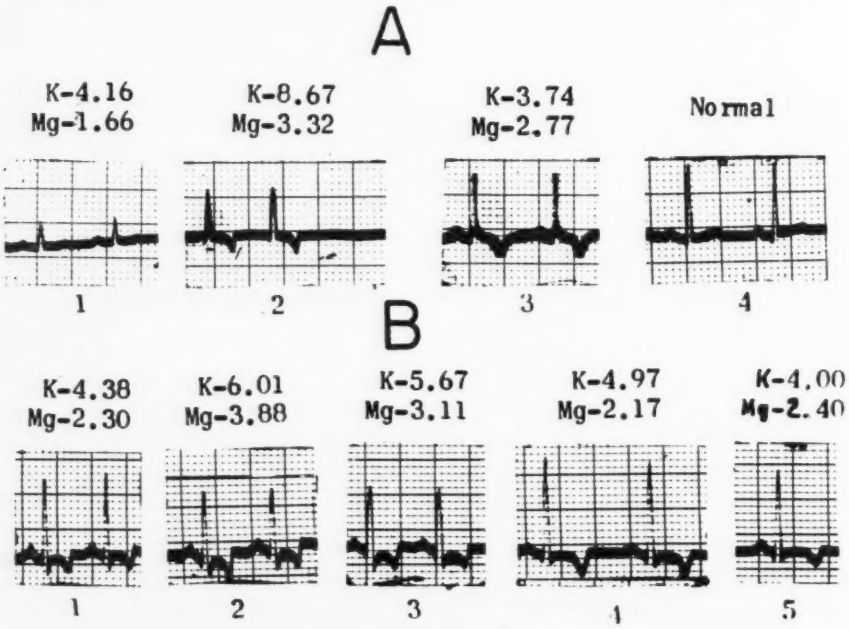

FIG. 2. Successive electrocardiographic tracings (lead II), of fasted adrenalectomized dogs developing insufficiency and during recovery on adrenal steroids. A. Animal treated with 10 mg. of 2-methyl FF. 1—Normal, DCA—maintained; 2—Insufficiency, absence of P waves and irregular ventricular rhythm; 3—24 hours after 2-methyl FF, regular rhythm but inverted T; 4—Normal, 7 days later. B. Treated with Aldosterone for 24 hours followed by 2-methyl FF. 1—Normal with inverted T*, DCA—maintained; 2—adrenal insufficiency, ST depression; 3—24 hours after Aldosterone—no improvement; 4—24 hours after 2-methyl FF, improvement, no ST depression; 5—48 hours after 2-methyl FF, rapid recovery.

* T inversion is not uncommon in ECG tracings of unoperated dogs.

was administered. The effect of DOC and 2-methyl FF upon plasma Mg, K and Na is shown in Figure 1.

An attempt was made to revive two dogs from insufficiency and maintain them in normal health with relatively huge (120 mg.), i.v. injections of progesterone given in divided doses three times daily. The results were equivocal since the dogs remained in a state of chronic insufficiency, i.e. were weak, spastic, had low blood pressures and abnormal electrolyte patterns with high Mg and K, low Na and Cl. Progesterone in the massive doses employed reduced the plasma Mg and K somewhat and elevated the plasma Na and Cl but the changes were insufficient to restore the normal electrolyte picture or relieve symptoms (Fig. 1). One animal died of adrenal insufficiency, the other, when verging on collapse, was restored to health by i.v. injections of 2-methyl FF.

DISCUSSION

Retention of an elevated plasma Mg for some days after disappearance of insufficiency symptoms and return of activity and vigor renders improbable the suggestion (4) that high Mg levels may be largely responsible for the metabolic disturbances characteristic of this condition. It seems not unreasonable to assume that the increased plasma Mg and K may represent a compensatory exchange of these primarily intracellular ions for some of extracellular constituents which apparently pass into the cells along with fluid during the period of cortical hormone deficit. Such an exchange would, to some small degree, restore to a more normal pattern the ionic imbalance of the extracellular fluid. The kidneys, owing to lack of mineralocorticoid e.g., aldosterone, retain the abnormal quantities of plasma Mg and K rather than excreting them promptly as would be the case with an intact dog possessing functional adrenal cortices or one treated with 2-methyl FF which is rich in both mineralo- and glucocorticoid activity.

Evidence is accumulating that abnormalities of the ECG exhibited by patients and animals with elevated plasma K are probably due more to a combination of factors such as low plasma Na, bicarbonate, pH and high Mg and K rather than to hyperkalemia *per se*. Roberts *et al.* (8) produced alterations of the ECG pattern of normal dogs by various means aside from increasing plasma K. The changes so induced were essentially similar in form and progression to those produced by elevated plasma. K.

ECG changes commonly occur in adrenal insufficiency (7, 8, 9) and cardiac arrythymia may be severe enough to cause collapse and death (10). The plasma electrolyte picture when ECG alterations are prominent, reveals high plasma Mg and K associated with low Na and Cl and plasma volume (Figs. 1, 2). Replacement therapy with glucocorticoids induces a profuse diuresis with renal elimination of surprising quantities of Na, Cl and K. The Na and Cl increase to normal values in the fasted dogs despite

the diuresis and continued loss of water and electrolyte. The increase in these plasma constituents is presumed due to a shift of fluid and certain electrolytes to the extracellular (and intravascular) space (11, 12). Plasma Mg apparently is excreted at a slower rate than K hence remains elevated after the latter cation has declined to its initial plasma level.

The ECG also shows deviations from normal for several days after plasma K has fallen to its control value. Although persistence of an abnormal ECG coincides with the period of higher than normal plasma Mg, other factors besides correction of the ionic and fluid imbalance between cells and the extracellular compartment probably contribute to the lag in response of the ECG.

Aldosterone, like DOC, exerts renal activity and induces excretion of K and Mg especially when followed with 24 hours by injection of a steroid (Fig. 1) rich in glucocorticoid activity. It also resembles DOC in its lack of properties requisite to shift fluid from the intra- and extracellular space of the fasted adrenalectomized dog exhibiting insufficiency. Internal redistribution of fluid apparently does not occur to any extent in DOC or aldosterone-treated animals. On the other hand the corticoids such as 1-dehydrohydrocortisone and 2-methyl-9α-fluorohydrocortisone enable these seriously ill animals to restore to normal their inspissated extracellular fluid volume, distorted plasma electrolyte picture, severe hemoconcentration and low arterial pressure (11, 12). The event of primary importance in this restoration process appears to be rehydration of the extracellular space by withdrawal of water and electrolytes from cells. Thus maintenance of a normal flow of fluid and salt between intra- and extracellular compartments is perhaps one of the principal functions of the so-called adrenal glucocorticoids whereas the activity of aldosterone appears to be chiefly confined to renal regulation of the salt and water content of this extracellular space.

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INACTIVITY OF ACID-EXTRACTED GROWTH HORMONE INJECTED IN AQUEOUS SUSPENSION

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ABSTRACT

The somatotropic activity of alkali- and acid-extracted growth hormone stored at various pH's was tested. The U.S.P. Growth Hormone Reference Standard, an alkali-extracted preparation, was found to lose most of its activity when stored at pH 3. Maximum stability was found at pH 11. No significant loss in activity was found between pH 5 and pH 11. Acid-extracted growth hormone showed the same activity at pH 3 or pH 11, but full activity was only found when the material was injected as a clear solution. Aqueous suspensions of acid-extracted growth hormone were found to be inactive. This effect was also evident after intravenous assay.

It is concluded that alkali-extracted growth hormone such as the U.S.P. Growth Hormone Reference Standard should never be exposed to pH's below 5 or above 11, while acid-extracted growth hormone, although not affected adversely by pH's as low as 3, should only be injected as a clear solution.

RECENTLY we reported that alkali-extracted growth hormone showed higher potency when assayed in solution at pH 8.0 than at pH 4.0, while acid-extracted preparations were not adversely affected by assay in acid (1). In the present report the optimal pH for storage of alkali-extracted growth hormone is established. It is also established that acid-extracted growth hormone while fully effective in solutions below pH 5.0, exhibits its activity only when administered as a clear solution. Aqueous suspensions of acid-extracted growth hormone have been found virtually inactive.

EXPERIMENTAL

The United States Pharmacopeia (U.S.P.) Growth Hormone Reference Standard (a highly purified alkali-extracted preparation obtained from bovine pituitaries) and acid-extracted growth hormone preparations obtained in our laboratory from pork or beef pituitaries by the method of Raben and Westermeyer (2) were used. Growth hormone activity was determined by 10-day growth tests in plateaued, male, hypophysectomized rats as previously described (3). In one experiment, a 15-day growth test in plateaued, female intact rats was used. Intact rats were considered to have plateaued by failure to grow 10 gm. in 20 days (4). The tibia test, also used, was carried out according to Greenspan *et al.* (5).

Effect of pH of Storage on Growth Hormone Activity

To study the effect of pH on alkali-extracted growth hormone, 5 mg. samples of U.S.P. Growth Hormone were dissolved into 5 ml. aliquots of 0.5% phenol water previously

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adjusted to various pH's from pH 3 to pH 12. After a period of 48 hours at 25° C all samples were diluted with 0.5% phenol to 100 μg. per ml. and adjusted to pH 8.5 with 0.1N NaOH or 0.1N HCl. The dilutions were assayed for growth hormone activity in hypophysectomized rats at a daily dose of 50 μg. During the 10-day course of the assay, all solutions were kept refrigerated. On the first day of the assay a *control* sample was prepared by making a fresh solution of U.S.P. Growth Hormone in 0.5% phenol at pH 8.5.

RESULTS

The results (Table 1) showed that U.S.P. Growth Hormone retained full activity at pH 11, and showed statistically insignificant losses when stored

TABLE 1. EFFECT OF pH ON GROWTH HORMONE ACTIVITY

pH treatment 48 hrs.—25° C	Number of hypophysecto-mized rats	Weight gain (gm.) per 10 days, mean ±S.E.	Appearance of injected test dilution	Difference from control
U.S.P. Growth Hormone Reference Standard (bovine, alkali-extracted)				
3	5	10.0±1.8	clear	P .05
5	5	16.6±1.3	clear	not sig.
7	5	16.6±1.1	cloudy	not sig.
9	5	16.8±1.1	clear	not sig.
11	5	20.6±1.0	clear	not sig.
12	4	1.0±0.6	clear	P .01
control	10	20.7±0.8	clear	—
Raben-type Growth Hormone (porcine, acid-extracted)				
3	6	17.5±1.7	clear	P .01
5	6	3.5±0.8	cloudy	not sig.
7	7	1.7±1.0	cloudy	not sig.
9	7	2.3±0.8	cloudy	not sig.
11	7	15.0±1.1	clear	P .01
12	6	0.3±0.4	clear	not sig.
control	4	1.2±1.0	cloudy	—

Control test dilutions prepared fresh on first day of 10-day test. All test dilutions kept and injected S.C. at pH 8.5. Daily dose: 50 μg. per rat.

at pH's between 5 and 11. Storage at pH 12 caused complete loss of activity and storage at pH 3 caused statistically significant loss. In the case of acid-extracted growth hormone storage at pH 12 likewise produced complete loss of activity, but unlike the U.S.P. material, all cloudy suspensions proved inactive. At the concentration of 1 mg. per ml. Raben-type hormone was not soluble at pH 5 to 9, and when dilutions were made from these and the pH adjusted to 8.5, the corresponding test dilutions remained cloudy. Activity was shown only by the solutions which had been sotred at pH 3 or 11, the dilutions of which appeared clear.

Inactivity of Acid-Extracted Growth Hormone Suspensions

The inactivity of acid-extracted growth hormone injected in aqueous suspension was further investigated in three other experiments. In the first experiment, a 10 mg. sample from each of two acid-extracted growth hormone preparations of different animal source was dissolved in 0.5 ml. of

TABLE 2. ACTIVITY OF BOVINE AND PORCINE RABEN-TYPE GROWTH HORMONE INJECTED IN SOLUTION AND IN SUSPENSION AT pH 8.5

Growth hormone preparation	Daily dose*	Weight gain (gm.) per 10 days, mean ±S.E.
Porcine		
Solution, pH 8.5	20 μg.	12.3±1.4
	80 μg.	24.0±2.5
Suspension, pH 8.5	20 μg.	3.8±1.1
	80 μg.	4.0±0.4
Bovine		
Solution, pH 8.5	100 μg.	16.6±3.0
Suspension, pH 8.5	100 μg.	8.6±3.0

* 0.5 ml. S.C., 4 hypophysectomized rats per group.

0.1N NaOH and this solution diluted with neutral 0.5% phenol water to injected concentrations. The pH of the final clear solutions was 8.5 ±0.3. A second 10 mg. sample of each preparation was suspended in 0.5 ml. of distilled water and this diluted with pH 8.5 phenol water. The latter test dilutions were cloudy suspensions but also had a final pH of 8.5 ±0.3. The results of this experiment (Table 2) demonstrate the inactivity of suspensions of acid-extracted growth hormone derived from porcine or bovine sources.

In the second experiment, the activity of acid-extracted growth hormone suspensions injected in alkaline and acid solution was tested. Clear solutions and cloudy suspensions were prepared as described using 0.05N HCl or 0.05N NaOH. The data (Table 3) show that aqueous suspensions are equally inactive at pH 3.5 or pH 9.0.

The third experiment was designed to establish if lack of activity of acid-extracted growth hormone suspensions was due to failure of absorption into the circulatory system from the site of subcutaneous injection. For this purpose, comparisons between suspensions and solutions of acid-extracted growth hormone were made following intravenous administration. Intravenous tests were made in hypophysectomized male, 100 gm. plateaued

TABLE 3. INACTIVITY OF ACID AND ALKALINE SUSPENSIONS OF RABEN-TYPE GROWTH HORMONE

Growth hormone injected in	Daily dose*	Weight gain (gm.) per 15 days, mean ±S.E.	Difference from control
Solution pH 9.0	1 mg.	23.5±1.1	P .01
Suspension pH 9.0	1 mg.	6.2±1.3	not sig.
Solution pH 3.5	1 mg.	25.8±1.6	P .01
Suspension pH 3.5	1 mg.	12.0±2.5	not sig.
(uninjected controls)	none	5.8±1.5	

* 1.0 ml. S.C., 5 intact rats per group.

rats. The animals were injected each day directly into the tail vein with 0.1 ml. volumes. Final body weights were determined on the day following the fourth daily injection, when the animals were sacrificed and their tibiae removed for determination of epiphyseal plate width. The results (Table 4) show that aqueous suspensions of acid-extracted growth hormone fail to produce their effect even when administered directly into the circulatory system.

COMMENT

Previous studies on the stability of alkali-extracted growth hormone have shown loss of activity following storage in acid (6, 7). In a previous report we have shown this to be the case for the recently released U.S.P. Growth Hormone Reference Standard (3). The present data demonstrate that the U.S.P. material is stable at pH 5 to 11, and that definite loss of activity takes place following storage at pH 3. These data are in agreement with those of Ellis *et al.* (7), who found their growth hormone preparation to have maximum stability between pH 5 and 11.5. It is of utmost importance then, that assay solutions of the current U.S.P. Growth Hormone Reference Standard should never have a pH lower than 5 or higher than 11.

Differences in the solubility of alkali-extracted and acid-extracted (Raben-type) growth hormone have been noted (8). Raben-type growth hormone is less soluble in aqueous solution than growth hormone obtained by methods using alkali extraction. At the concentrations used here, Raben-type growth hormone was not soluble at pH's between 5 and 9, and the resulting aqueous suspensions were found inactive. The question of whether inactive suspensions can be brought back into solution and, if so, whether the solutions show activity has not been fully investigated, but from what data is available it appears that material which has stood for 24 hours in suspension can be redissolved by changing the pH, and the activity regained. In any event, the present report shows that it is essential to obtain complete solution of acid-extracted growth hormone in the vehicle being injected if the full activity of the hormonal preparation is to be obtained.

It is not clear why aqueous suspensions of acid-extracted growth hor-

TABLE 4. INACTIVITY OF A RABEN-TYPE GROWTH HORMONE SUSPENSION ADMINISTERED INTRAVENOUSLY

Growth hormone injected in	Weight gain (gm.)* per 4 days, mean ±S.E.	Width of epiphyseal plate (micrometer units) mean ±S.E.
Solution pH 4.0	7.2±0.4	43±2.0
Suspension pH 4.0	0.2±0.1	29±1.0
Untreated Controls†	0.0±0.9	15±2.0

* Daily dose: 100 μg. I.V., 4 hypophysectomized rats per group.
† Data from cumulative records with the colony of rats.

mone should prove inactive. From the present data, it is clear, however, that it it is not due to lack of absorption of the suspended particles from the site of injection. Aqueous suspensions of acid-extracted growth hormone have been shown to be inactive even when assayed by the intravenous route. The inactivity of these preparations may simply mean that the particulate matter is being filtered out.

Acid-extracted growth hormone preparations have been shown to have the same activity after storage at pH 2.7 or pH 11 (9). This wide range of pH in which the Raben-type growth hormone retains its activity simplifies the making of proper solutions for its injection. In the present investigation, acid-extracted growth hormone was dissolved in either dilute acid or dilute alkali, and assay dilutions made with distilled water or 0.5% phenol.

Acknowledgment

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THE ROLE OF GROWTH HORMONE IN CALORIGENESIS AND THYROID FUNCTION[1]

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ABSTRACT

Experimental evidence is presented which demonstrates that growth hormone intensifies the characteristic action of thyrotrophin on calorigenesis in hypophysectomized rats, presumably through augmentation of thyroid function as measured by thyroid weight, morphology and I^{131} uptake. Growth hormone also magnifies the calorigenic action of thyroxine in hypophysectomized rats indicating that the enhancement of calorigenesis by growth hormone is partly peripheral. Excessive heat production in hypophysectomized rats resulting from the administration of high levels of either thyrotrophin or thyroxine with growth hormone is not compatible with life in the absence of adrenal glucocorticoids.

IT HAS previously been shown that the calorigenic decline is more abrupt after hypophysectomy than after thyroidectomy (1). Thyroxine and triiodothyronine were also shown to be calorigenically more effective in thyroidectomized than in hypophysectomized rats. Thyrotrophin (TSH) administered to the hypophysectomized rat, in doses sufficient to produce histological evidence of thyroid hyperactivity, increased but did not restore the metabolic rate to normal. These findings indicated that the anterior pituitary exerts an effect on the gaseous metabolism in addition to its secretion of TSH. This effect was ascribed tentatively to adrenocorticotrophic hormone (ACTH) acting indirectly through adrenal steroid production. The reasons given were:

1. ACTH and adrenal glucocorticoids were calorigenic in hypophysectomized, thyroidectomized, and hypophysectomized-thyroidectomized rats. Thyroidectomized-adrenalectomized rats maintained by either NaCl or low doses of adrenal steroids did not respond calorigenically to ACTH.[2] The melanocyte stimulating hormone and lactogenic hormone, previously implicated as calorigenic hormones, did not increase oxygen consumption in hypophysectomized rats.

2. Neither TSH nor thyroxine influenced the atrophic adrenals of the

[1] Supported in part by U. S. Public Health Service grant A-800(C_2) and by American Cancer Society, California Division grant 150(C_2D).

[2] Recent unpublished experiments have also shown that hypophysectomized-adrenalectomized rats maintained by low doses of desoxycorticosterone acetate and hydrocortisone acetate do not rerespond calorigenically to ACTH.

hypophysectomized rat, whereas thyroxine in the thyroidectomized rat restored the adrenals. In normal rats overconveyal of thyroxine increased heat production above normal and produced adrenal cortical hypertrophy.

3. Following hypophysectomy there is necessarily an immediate and complete loss of ACTH whereas after thyroidectomy the titer of circulating ACTH presumably is reduced more gradually without complete loss. This is supported by the observation that thyroidectomy changes in the anterior pituitary require at least two weeks to become pronounced and that the adrenals are not as atrophic following thyroidectomy as after hypophysectomy. The administration of hydrocortisone to the thyroidectomized rat causes further regression of the adrenals to levels approaching that of the hypophysectomized rat, which is interpreted as evidence for further suppression of ACTH release. The atrophic adrenals of the thyroidectomized rat respond remarkably well to exogenous ACTH, indicating that their atrophy after thyroidectomy is a consequence of thyroxine deficiency at the level of the anterior lobe.

It is well known that thyroxine, in addition to repairing the adrenals, restores the growth of thyroidectomized rats through reinstatement of growth hormone (GH) secretion by the anterior pituitary, and by intensifying the action of GH at the periphery. The possibility exists, therefore, that thyroxine is calorigenically more effective in the thyroidectomized than in the hypophysectomized rat because it initiates secretion of GH. The immediate loss of GH following hypophysectomy could also explain the more rapid decline in heat production as compared to thyroidectomy. After thyroidectomy the decline in growth rate exactly parallels the decline in metabolic rate. The experimental evidence to support the contention that GH is also a factor in determining the levels of heat production in the rat will now be presented.

MATERIAL AND METHODS

In this investigation normal, hypophysectomized, thyroidectomized and hypophysectomized-thyroidectomized male and female rats of the Long-Evans strain were employed. Hypophysectomy and thyroidectomy were performed at 28 days of age. In rats subjected to both operations, hypophysectomy was at 28 days of age and thyroidectomy 21 days later. The hormones which have been examined alone and in combination were TSH, GH, *l*-thyroxine and hydrocortisone acetate. Hormonal treatment was begun 21–28 days after hypophysectomy and continued 14 days unless otherwise indicated. Injections in the thyroidectomized rats were given 45 days after operative intervention and continued for 16 days. Operative procedures, criteria of completeness of ablations, care of animals and types of hormones injected are given in more detail in the appendix.

The metabolic rates were determined eight hours after the last hormone injection with a closed circuit respiration apparatus consisting of eight chambers which was patterned after that described by Kleiber (6). In order to control variations incident to time of day or night in this nocturnal animal, the metabolic rates were measured simultaneously for representatives of the treated and control groups. All rats were fasted four hours, then allowed to adjust to the temperature of the apparatus ($30 \pm 0.1°$ C) for one hour

prior to oxygen consumption measurements. The liters of oxygen consumed per rat over a 2.5 hour experimental period were converted into calories per hour by the factor 4.80. The calories per hour per square meter of body surface were calculated by Benedict's formula for the rat:

$$\text{Area in } m^2 = \frac{9.0^3\sqrt{(\text{Body Weight in gm.})^2}}{10{,}000}$$

The metabolic rate so described is the standard metabolic rate and not the basal metabolic rate as defined for human beings.

RESULTS

Thyrotrophin:

It can be seen from Table 1 that 2 mg. per day of a purified TSH preparation (MED 5 μg. total dose in the 4 day test) was required in order to reestablish a normal metabolic rate in the hypophysectomized rat. Histologically, the thyroid showed almost complete resorption of colloid at doses of 500 μg. and above (Fig. 3). Although the absolute weight of the gland remained below normal at all doses, the gland weight expressed per 100 gms. of body weight exceeded normal at doses of 500 μg. and above. I^{131} uptake by the thyroid of the hypophysectomized rat did not exceed normal at the highest dose level as it did when TSH was administered to normal animals (Fig. 1).[3] After treatment with 2 mg. of TSH I^{131} uptake expressed per mg. of thyroid was increased to the same extent in normal and hypophysectomized rats.

Growth Hormone:

In earlier efforts to determine whether GH had a calorigenic action, in which relatively crude GH was administered to normal animals, less calori-

TABLE 1. CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED FEMALE RATS TO THYROTROPHIN*

Group	Dose, μg./da./ 14 da.	No. of rats	Metabolic rate, Cal./m.²/ hr.	Thyroid		Body weight, gm.	
				Weight, mg.	18 hr. I^{131} uptake‡	Initial	Final
H̄ Injected	5	6	20.9±0.9†	6.5±0.4	1.3±0.4	66	71
	10	6	22.6±0.6	6.5±0.4	1.1±0.4	67	74
	25	6	24.1±0.5	7.1±0.5	2.2±0.4	67	75
	50	6	23.7±0.8	7.3±0.7	1.8±0.9	67	75
	100	11	28.7±1.4	7.5±0.6	5.9±0.9	70	79
	500	10	31.4±1.0	8.3±0.6	7.3±0.7	72	81
	1000	6	34.8±1.6	9.6±0.7	9.3±1.4	73	82
	2000	6	39.9±3.1	10.8±0.9	9.3±1.4	73	81
H̄ Control	—	41	21.2±0.4	5.8±0.2	0.6±0.1	69	72
N Control	—	20	38.1±1.2	15.1±1.3	9.5±2.1	175	197

* MED 5 μg. (see appendix).
† Figures preceded by ± in the tables are the standard errors of the means.
‡ Expressed as per cent of injected dose in all tables.

[3] This was not due to the interval selected as was shown by studies of I^{131} uptake as a function of time after TSH administration. It was found that I^{131} uptake was still maximal 18 hours following the administration of a tracer dose.

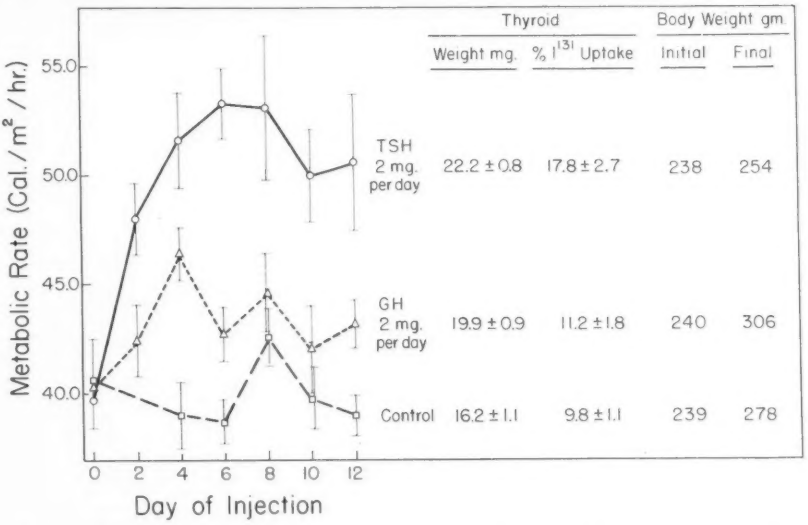

FIG. 1. Calorigenic response of normal male rats 64 days old at onset, to TSH and GH (G-28). Each point on these curves was based on determination employing 8 rats. The vertical bars extending from the points on the curves in all figures represent the standard errors of the means.

genic action was observed after thyroidectomy; the higher calorigenesis in normal animals was therefore attributed to thyroid mediation (7, 8). Figure 1 shows the small but definite calorigenic action of purified GH in intact animals.

The three purified GH preparations used here also possessed recognizable calorigenic activity in hypophysectomized rats at daily dose levels of 25 μg. and above (Table 2 and Fig. 2). The calorigenic response of hypophysectomized rats to 500 μg. of GH in fact equalled that from 500 μg. of TSH.[4] There was, however, a marked difference in the histological response of the thyroids as shown in Figure 3. Part of this increase in heat production from GH was attributable to TSH contamination, inasmuch as the thyroid weight, histology, and I^{131} uptake showed a minimal increase at the 100 μg. dose, and were definitely increased at the 500 μg. level. The influence

[4] Although the metabolic rates of only 10 hypophysectomized female rats are shown in Table 1 at the 500 μg. dose level for TSH (G-94), a total of 57 male and female hypophysectomized rats, 21–28 days postoperative at onset, were treated with this same preparation at this dose level for 14 days. The average metabolic rate was 29.4 ± 0.4 Cal./m.²/hr. The results from only 18 hypophysectomized male rats are shown in Table 2 at the 500 μg. level of GH. The several purified growth hormone preparations were actually administered to a total of 36 male and female hypophysectomized rats at this dose level and under conditions identical to those used in thyrotrophin administration. The average metabolic rate was 28.5 ± 0.4 Cal./m.²/hr. The value for 76 male and female hypophysectomized controls was 20.2 ± 0.3 Cal./m.²/hr.

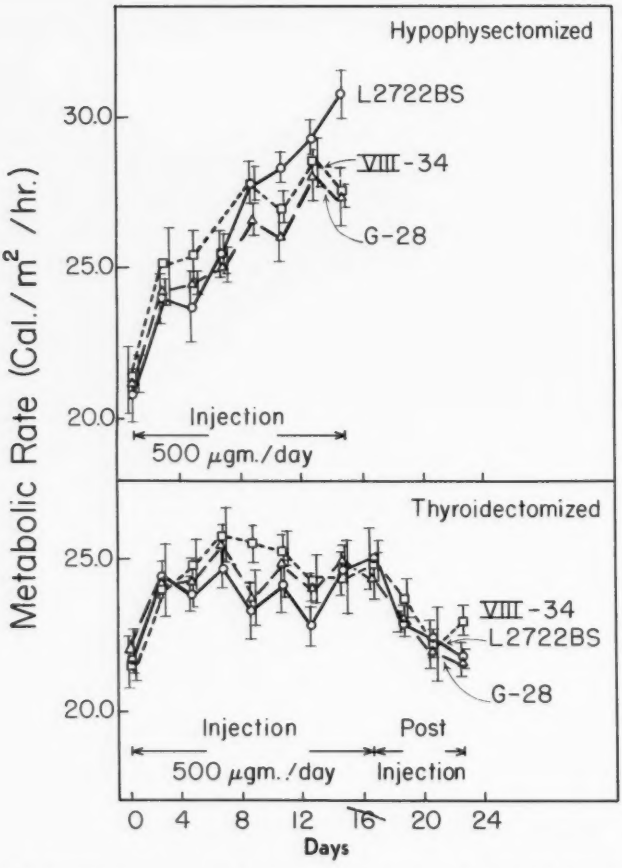

FIG. 2. Calorigenic response of hypophysectomized and thyroidectomized rats to several GH preparations. Each point on these curves was based on determinations employing 6 rats. Complete data on these rats is shown in Table 2 at the 500 μg. dose level for each preparation.

of TSH contamination in the GH preparations was further shown by their smaller calorigenic action in thyroidectomized than in hypophysectomized rats (Table 2 and Fig. 2). Both types of animal initially had the same low metabolic rate but the hypophysectomized rat possessed a thyroid gland which could respond to TSH and, therefore, gave a greater increase in metabolic rate than did the thyroidectomized rat.

A greater increase in thyroid weight occurred in hypophysectomized rats after prolonged GH administration as can be seen by comparing Tables 2 and 3 showing results from 2 weeks and 6 months. Histologically, the thyroids after chronic treatment showed extremely large follicles with dense

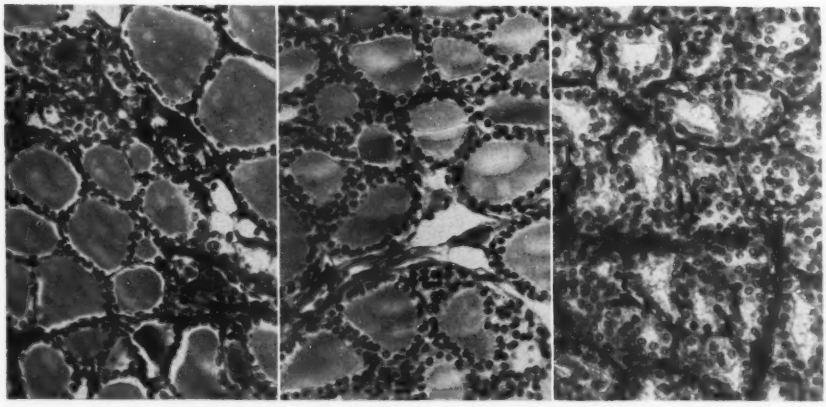

FIG. 3. Thyroids of hypophysectomized rats. Iron hematoxylin and aniline blue ×500.

Left: Untreated hypophysectomized rat.

Center: From hypophysectomized rat injected with 500 μg. GH (L2722BS) daily for 14 days.

Right: From hypophysectomized rat injected with 500 μg. TSH daily for 14 days.

TABLE 2. CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED AND OF THYROIDECTOMIZED RATS TO GROWTH HORMONE PREPARATIONS*

Preparation	Dose, μg./da./ 14 da.	No. of rats	Metabolic rate, Cal./m.²/ hr.	Thyroid		Body weight, gm.	
				Weight, mg.	18 hr. I^{131} uptake	Initial	Final
VIII-34 H ♀	10	4	21.5±0.6	6.1±0.5	0.5 ±0.2	70	86
	25	6	25.9±1.3	6.6±0.6	0.7 ±0.1	70	92
	50	6	26.8±1.1	8.0±0.8	0.6 ±0.1	71	97
	100	18	25.6±0.5	7.0±0.6	1.2 ±0.2	71	101
H ♂	500	6	27.4±0.4	8.0±0.4	1.5 ±0.6	78	119
T† ♂	500	6	24.4±1.2		0.01±0.0	134	160
G-28 H ♂	50	5	25.0±0.8	5.4±0.4	0.5 ±0.1	66	98
	100	4	25.5±0.3	6.5±0.3	0.7 ±0.1	77	118
	500	6	27.3±0.9	8.5±0.8	1.9 ±0.3	78	118
T ♂	500	6	24.9±0.6		0.01±0.0	134	164
L2722BS H ♂	50	5	24.1±0.2	7.3±0.4	0.7 ±0.1	74	110
	100	6	26.4±0.7	7.6±0.4	1.3 ±0.4	65	97
	500	6	30.7±0.7	10.6±0.5	3.9 ±0.3	78	123
T ♂	500	6	24.5±0.7		0.03±0.0	134	163
N ♀ Control	—	20	38.1±1.2	15.1±1.3	9.5 ±2.1	175	197
N ♂ Control	—	35	39.8±0.6	16.7±0.5	11.1 ±0.8	247	287
H ♀ Control	—	41	21.2±0.4	5.8±0.2	0.6 ±0.1	69	72
H ♂ Control	—	35	19.3±0.3	6.3±0.1	0.5 ±0.1	83	87
T ♂ Control	—	18	21.7±0.4		0.01±0.0	134	138

* MED for all preparations 30 μg. (see appendix).
† Forty-five days post-thyroidectomy at onset.

TABLE 3. INCREASED THYROID WEIGHT IN HYPOPHYSECTOMIZED FEMALE RATS AFTER CHRONIC GROWTH HORMONE (G-43) ADMINISTRATION

Treatment	Dose, μg./da./ 180 da.	No. of rats	Metabolic rate, Cal./m.²/ hr.	Thyroid		Body weight, gm.	
				Weight mg.	18 hr. I^{131} uptake	Initial	Final
Growth Hormone	200	9	22.2±0.9	17±1.3	0.3±0.1	71	284
H Control	—	5	20.8±0.2	8±0.2	0.5±0.1	73	100
N Control	—	5	30.0±1.0	26±3.1	9.2±0.6	134	290

colloid and flattened inactive epithelium. Interfollicular connective tissue was increased above that characteristic of either the normal or hypophysectomized control of the same age. The low I^{131} uptake offered additional confirmatory evidence as to the inactivity of the gland. Although bovine TSH stimulates the thyroid acutely, the gland eventually becomes refractory and returns to hypophysectomized controls levels after 50 days. Since there was no evidence of thyroid stimulation after chronic administration of GH the increased gland weight, due to colloid storage and to increased connective tissue, was attributed to a GH action.

That GH is itself calorigenic has been shown by its action in hypophysectomized rats at dose levels that did not stimulate the thyroid, and by persistence of its calorigenic action (although reduced) in thyroidectomized rats. As confirmatory evidence that the calorigenic activity of GH was not entirely attributable to TSH contamination, hypophysectomized rats were injected with GH that had been treated with sodium metaperiodate to destroy TSH by oxidation of its carbohydrate moiety (9). The authors are indebted to Irving I. Geschwind for supplying the periodate treated GH. After periodate treatment morphological evidence of low grade thyroid activity at the 500 μg. level was no longer present, and the I^{131} uptake was reduced to control levels although the calorigenic response was only slightly impaired (Table 4). It was of interest that an increase in gland weight still occurred after oxidative destruction of TSH. Further confirmatory evidence that the calorigenic activity is a property of GH has been obtained from preliminary experiments done in collaboration with Tetsuo Hayashida. These experiments have shown that the simultaneous injection of GH with rabbit anti-serum, containing specific antibodies to GH,

TABLE 4. CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED MALE RATS TO PERIODATE TREATED GROWTH HORMONE (L2722BS)

Treatment	Dose, μg./ da./14 da.	No. of rats	Metabolic rate, Cal./m.²/ hr.	Thyroid		Body weight, gm.	
				Weight, mg.	18 hr. I^{131} uptake	Initial	Final
Growth Hormone	500	6	28.8±0.8	8.6±0.8	1.5±0.2	79	129
Periodate Treated Growth Hormone	500	5	26.0±0.5	8.4±0.6	0.5±0.1	76	108
H Control	—	5	20.4±0.4	6.1±0.5	0.5±0.1	74	79

TABLE 5. CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED FEMALE RATS TO THYROTROPHIN AND GROWTH HORMONE (VIII-34), ALONE AND IN COMBINATION

Treatment	Dose, μg./da./ 14 da.	No. of rats	Metabolic rate, Cal./m.²/ hr.	Thyroid		Body weight, gm.	
				Weight, mg.	18 hr. I^{131} uptake	Initial	Final
Thyrotrophin	100	5	27.1 ± 2.8	6.0 ± 0.6	5.8 ± 1.6	69	83
Growth Hormone	100	5	24.2 ± 1.1	5.4 ± 0.5	1.7 ± 0.4	69	96
Combination	100 + 100	5	36.1 ± 2.5	10.4 ± 0.9	13.4 ± 0.9	67	109
H Control	—	5	19.3 ± 0.9	5.1 ± 0.3	1.2 ± 0.7	66	68
N Control	—	20	38.1 ± 1.2	15.1 ± 1.3	9.5 ± 2.1	175	197

destroyed the calorigenic action of the hormone in hypophysectomized rats (3).

Combination of Thyrotrophin and Growth Hormone:

As large doses of TSH (1–2 mg.) were required to raise the metabolic rate of the hypophysectomized rat to normal, and as GH increased heat production, the possibility of an additive or synergistic action between the two hormones was investigated. The data presented in Table 5 and Figure 4 demonstrate that GH increased the calorigenic activity of TSH. It also intensified the characteristic effect on thyroid weight and I^{131} uptake. (When the gland weights given in Figure 4 are expressed in mg. per 100 gms. of body weight, and the I^{131} uptake per mg. of thyroid, then the values are the same for the rats receiving the combination as for those receiving TSH alone.) The increased metabolic rate from the combination could not

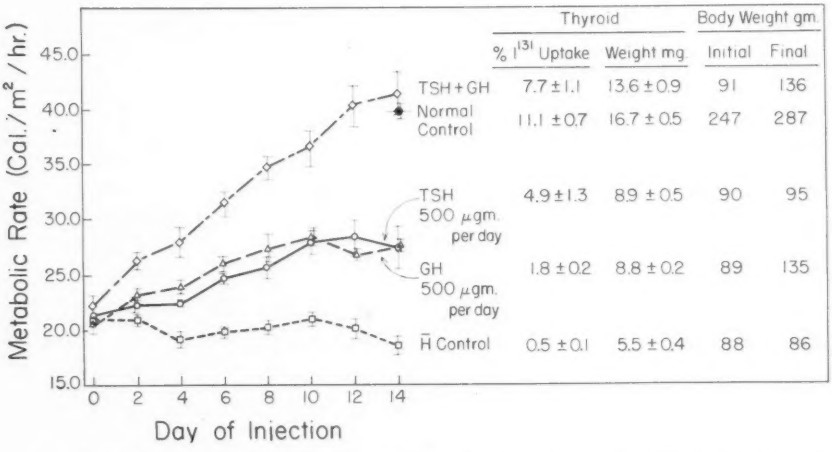

FIG. 4. Calorigenic response of hypophysectomized male rats to TSH and GH (G-28), alone and in combination. Each point on these curves was based on determinations employing 6 rats. There were 34 normal male rats at the single terminal point.

be attributed to TSH contamination in the GH because the contamination would not be of significance in the presence of these large doses of TSH. The thyroids of the group receiving the combined treatment appeared more active than after TSH alone both from their morphological characteristics and from the increased mitotic rate.[5] The failure of TSH to augment the action of growth hormone on body weight (Fig. 4) is interpreted as due to the high dose level of TSH injected with the consequent high metabolism.

Combination of Thyroxine and Growth Hormone:

The calorigenic effectiveness of thyroxine in the hypophysectomized rat was also increased by the simultaneous administration of GH (Table 6).

TABLE 6. CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED AND OF HYPOPHYSECTOMIZED-THYROIDECTOMIZED RATS TO THYROXINE AND GROWTH HORMONE (VIII-34), ALONE AND IN COMBINATION

Group	Treatment	Dose, μg./da./14 da.	No. of rats*	Metabolic rate, Cal./m.²/hr.	18 hr. I^{131} uptake	Body weight, gm.	
						Initial	Final
H ♀	Thyroxine	1	6	25.7±1.0	0.4±0.0	69	82
		3	6	30.8±1.2	0.4±0.1	73	88
		5	6	38.2±1.9	0.3±0.0	72	89
	Growth Hormone	100	5	26.8±1.0	1.0±0.1	71	104
	Combination	1+100	6	33.3±1.0	0.7±0.1	69	110
		3+100	5	38.4±2.5	0.5±0.1	69	103
		5+100	4	47.0±2.6	0.5±0.1	73	115
H ♂	Thyroxine	1	6	27.0±1.1	0.3±0.0	79	84
		3	6	33.5±0.6	0.2±0.0	85	95
	Growth Hormone	100	6	23.8±0.5	0.7±0.2	77	102
	Combination	1+100	6	36.1±0.7	0.4±0.1	75	117
		3+100	6	45.0±1.9	0.4±0.1	81	118
H † ♀	Thyroxine	1	4	24.5±0.7	0.04±0.0	75	82
		3	3	30.9±0.9	0.01±0.0	72	79
	Growth Hormone	100	1	25.4	0.02	83	100
	Combination	1+100	3	35.2±1.9	0.02±0.0	71	94
		3+100	2	39.7±0.7	0.02±0.0	68	90
N ♀ Control	—	—	20	38.1±1.2	9.5±2.1	175	197
N ♂ Control	—	—	35	39.8±0.6	11.1±0.8	247	287
H ♀ Control	—	—	41	21.2±0.4	0.6±0.1	69	72
H ♂ Control	—	—	35	19.3±0.3	0.5±0.1	83	87
HT ♀ Control	—	—	2	20.8±2.0	0.02±0.0	71	70

* Six animals were present at onset in each injected group.
† Twenty-two days post-hypophysectomy and one day post-thyroidectomy at onset.

[5] The mitotic figures were counted in a single 8 μ central section of the thyroid from each of 6 rats, in each group, and were expressed as average number of mitoses per section per group: TSH = 5, GH = 2, TSH + GH = 22, hypophysectomized control = 0.

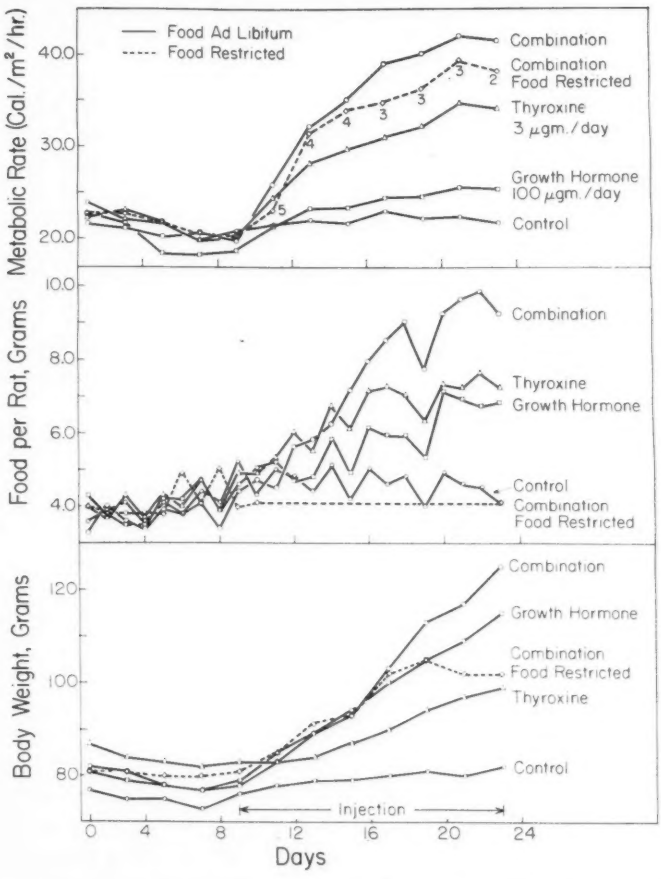

FIG. 5. Effect of food restriction on the calorigenic response of hypophysectomized male rats to thyroxine and GH (G-28) in combination. Each point on these curves was based on determinations employing 5 rats except where indicated by number on the curves. The rats were housed in individual cages and fed only a wet mash of the standard laboratory diet.

The combination of 5 μg. of thyroxine and 100 μg. of GH in hypophysectomized female rats produced an increase in heat production which was not compatible with life as evidenced by the mortality in this group. In the second experiment in which hypophysectomized males were used, the dose of thyroxine was reduced to 3 μg. and all animals receiving this dosage in combination with GH survived although the metabolic rate exceeded normal. In order to eliminate any small increase in thyroid activity attributable to GH, hypophysectomized-thyroidectomized rats were treated with the combination of thyroxine and GH. The increased effectiveness of the

combined treatment could again be seen though the data was limited because of the high mortality (Table 6). The death rate was greatest in those experimental groups in which the metabolic rate was increased the most.

Food consumption was increased in hypophysectomized rats injected with the combination of thyroxine and GH (Fig. 5). It was possible therefore, that this increased food consumption was an integral part of the reaction leading to increased calorigenesis. For this reason heat production was measured in hypophysectomized rats in which the combination was given and in which food intake was restricted to pretreatment levels. It can be seen from Figure 5 that food restriction reduced only slightly the heat production resulting from the injection of the combination of thyroxine and GH. The severity of food restriction resulted in progressively increased mortality as body stores of energy were exhausted.

Additional evidence that GH intensifies the calorigenic action of thyroxine was obtained from pituitary transplantation experiments in collaboration with Irving I. Geschwind. As shown by other workers (10, 11), pituitaries transplanted under the kidney capsule or to the anterior chamber of the eye of hypophysectomized rats retain the ability to secrete GH although at reduced levels. This has been confirmed as shown in Figure 6. The injection of thyroxine, beginning the day of hypophysectomy and transplantation, both augmented body growth and intensified calorigenesis above that which characterized animals with grafts but untreated with thyroxine, or animals without grafts treated with thyroxine. Thyroid weight, histology and I^{131} uptake, and adrenal weight were at the hypophysectomized control level in the rats bearing transplants and receiving thyroxine. In view of the atrophic endocrine organs, the increased effectiveness of thyroxine on the metabolic rate of hypophysectomized rats possessing transplants could be attributed to GH secretion by the grafts. Full details of these experiments will be presented in a later publication by Dr. Geschwind.

2,4-dinitrophenol (DNP) is another potent calorigenic agent which, like thyroxine, stimulates ATP-ase activity (12), interferes with oxidative phosphorylation (13), and prevents thyroidectomy changes in the pituitary (14). It was considered possible that DNP therefore, might act synergistically with GH on heat production in a manner similar to thyroxine. The calorigenic action of DNP requires only minutes to become manifest, whereas thyroxine, at low dosage, requires two weeks for a maximum reponse. Growth hormone also requires some time for the full effect. For this reason hypophysectomized rats were pretreated for 15 days with GH and then given a single subcutaneous injection of DNP in a dosage of 10 mg. per kg. of body weight. Heat production was determined immediately prior to and after injection of DNP. It can be seen from Table 7 that GH produced a definite intensification of calorigenesis from DNP.

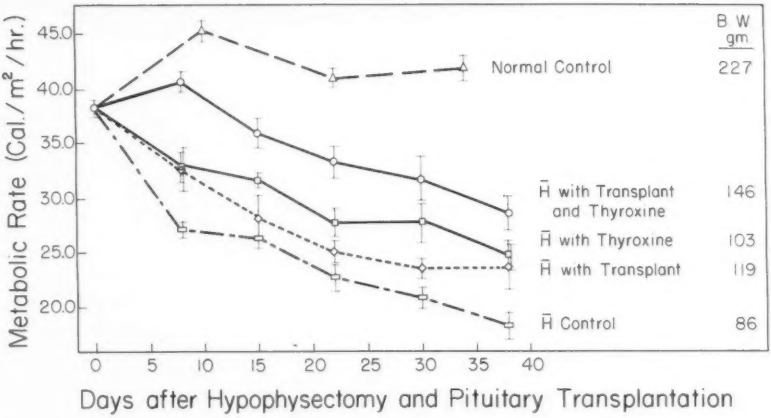

FIG. 6. Increased calorigenesis with thyroxine in hypophysectomized male rats possessing pituitary transplants. Four pituitaries were transplanted under the left kidney capsule on the day of hypophysectomy. There were 24 normal rats, 3 hypophysectomized rats with transplants and thyroxine, 5 hypophysectomized rats with thyroxine, 3 hypophysectomized rats with transplant, and 5 untreated hypophysectomized controls at each point on the curves. The solid lines represent the hypophysectomized rats receiving 3 μg. *d-l* thyroxine for 7 days and then 1.5 μg. *l*-thyroxine for the remaining 31 days.

Combinations of Hydrocortisone, Growth Hormone, and Thyroxine:

Since GH had been shown to intensify the action of well known calorigenic agents (TSH, thyroxine, and DNP), and since hydrocortisone was known to be calorigenic in hypophysectomized rats, the ability of GH to increase the calorigenic action of hydrocortisone was also tested. Table 8 shows that no increase in respiratory metabolism above that resulting from hydrocortisone alone occurred from the combination, although GH did intensify the effects of hydrocortisone on thyroid I^{131} uptake and histological structure. However, when hydrocortisone was combined with GH and thyroxine heat production was increased on day 21 to a remarkable level for hypophysectomized animals, and without loss of life (Table 9). (Determinations were also made on day 12 of the injection period at which time the metabolic rate was 70.0 Cal./m.²/hr.).

TABLE 7. CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED MALE RATS* TO GROWTH HORMONE (G-28) AND DINITROPHENOL IN COMBINATION

Treatment	Dose, μg./da./ 15 da.	No. of rats	Final body weight gm.	Metabolic rate, Cal./m.²/hr.	
				Pre-DNP	Average during 3 hrs. post-injection of 10 mg. DNP/kg. B.W.
Growth Hormone	100	6	125	25.8 ± 0.3	33.4 ± 1.1
H̄ Control	—	6	83	20.1 ± 0.7	24.7 ± 0.5

* 75 days old and 47 days post-operative at injection of DNP.

TABLE 8. CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED FEMALE RATS TO GROWTH HORMONE (VIII-34) AND HYDROCORTISONE ALONE, AND IN COMBINATION

Treatment	Dose, μg./da./14 da.	No. of rats	Metabolic rate, Cal./m.²/hr.	Thyroid		Body weight, gm.	
				Weight mg.	18 hr. I^{131} uptake	Initial	Final
Growth Hormone	100	5	25.3±0.5	6.5±0.2	1.2±0.3	72	102
Hydrocortisone	500	5	33.3±2.0	5.2±0.5	1.9±0.4	70	59
Combination	100+500	5	33.4±2.3	6.0±0.9	2.9±0.6	71	73
H Control	—	5	21.5±0.4	4.8±0.5	0.6±0.0	70	71

The preceding experiment was repeated using all combinations of the three hormones in order to determine the relative importance of each. As shown in Table 10 metabolic rates were determined on day 13 of the injection period and on day 27, two days prior to autopsy. Excessive heat production in the hypophysectomized rats receiving thyroxine and GH, with no adrenal steroids, had already reduced the number of animals from 6 to 4 by day 13. During this determination one of the four rats died in the apparatus with a metabolic rate of 70.5 Cal./m.²/hr. Hydrocortisone by day 13 did not intensify calorigenesis over that given by thyroxine and GH alone, however, it did increase the survival to the end of the 29-day injection period. In this series also, GH intensified the action of hydrocortisone on thyroid I^{131} uptake and histological structure. It was concluded that GH and thyroxine were the principal synergists and that hydrocortisone permitted observation of the synergism through maintenance of life.

DISCUSSION

The results show that low doses of purified GH produced some increase in heat production in the hypophysectomized rat which was not accompanied by evidence of thyroid stimulation. GH also produced a small increase in the metablic rate of thyroidectomized and of hypophysectomized-thyroidectomized rats. Periodate oxidation of the TSH contamination in growth hormone did not destroy the calorigenic action of GH. These findings suggest that the increases in heat production in hypophysectomized rats from GH are not entirely attributable to TSH contamination. In Table 11 is presented a comparison of the minimal effective dose of TSH, GH, and ACTH for calorigenesis and thyroid stimulation in hypophysectomized rats. This summary serves to emphasize again that heat produc-

TABLE 9. CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED FEMALE RATS TO THYROXINE, HYDROCORTISONE, AND GROWTH HORMONE (VIII-34), ALONE AND IN COMBINATION

Treatment	Dose, μg./da./21 da.	No. of rats	Metabolic rate, Cal./m.²/hr.
Thyroxine (Tx)	5	5	39.9±1.3
Hydrocortisone (F)	250	3	25.8±0.1
Tx+F+GH	5+250+500	4	67.4±5.9
H Control	—	4	21.0±0.9

TABLE 10. CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED FEMALE RATS TO THYROXINE, HYDROCORTISONE, AND GROWTH HORMONE (VIII-34), ALONE AND IN COMBINATION

Treatment	Dose, μg./da./ 27 da.	No. of rats		Metabolic rate, Cal./m.²/hr.		Thyroid		Body weight, gm.	
		13 da.	27 da.	13 da.	27 da.	Weight, mg.	18 hr. I^{131} uptake	Initial	Final
Thyroxine (Tx)	5	6	6	38.6±1.2	39.8±1.2	6±0.4	0.2±0.0	66	91
Hydrocortisone (F)	250	6	6	30.8±1.4	30.3±1.7	7±0.6	1.9±0.3	70	63
Growth Hormone (GH)	500	6	6	29.9±1.0	26.9±0.7	13±1.1	0.9±0.3	70	156
Tx+F	5+250	6	3	41.8±1.3	42.3±1.8	6±0.5	0.7±0.1	71	63
Tx+GH	5+500	4	3	54.2±6.3	46.9±1.3	11±2.1	0.4±0.1	75	197
F+GH	250+500	6	3	31.6±0.8	30.5±1.4	10±1.2	3.6±0.6	66	116
Tx+F+GH	5+250+500	6	5	50.0±1.3	55.9±2.0	10±0.4	0.9±0.3	72	121
Control	—	6	5	21.1±0.9	20.4±0.5	7±0.7	0.6±0.1	71	78

tion from either GH or ACTH occurs at doses which give no evidence of thyroid stimulation.

High doses of TSH were required to restore the metabolic rate and I^{131} uptake of the hypophysectomized rat to normal although at these doses the thyroid was hyperplastic. The weight of the thyroid was not restored to normal by the highest dose level employed (400 times the MED). Equally effective stimulation resulted from considerably lower doses of TSH administered in combination with GH. As GH increases calorigenesis from thyroxine in hypophysectomized rats, the increase in calorigenesis with TSH must also be due partly to augmentation of the action of thyroxine peripherally. The relative importance of the peripheral action of GH, and its role in increasing calorigenesis through increase in thyroid activity remains to be determined.

Although TSH increases thyroid weight and I^{131} uptake in hypophysectomized rats, it does not increase them above normal even at high doses. Inasmuch as body weight is not increased, thyroid weight does exceed normal when expressed in terms of body weight. Upon the addition of GH to TSH thyroid weight is further increased, however, since growth occurs, thyroid weight expressed per 100 gms. of body weight does not exceed that achieved with TSH alone. The I^{131} uptake is increased more than the thyroid weight after TSH and therefore, I^{131} uptake expressed per mg. of thyroid is increased above normal. Upon addition of GH the increase only parallels the thyroid weight increase and therefore, I^{131} expressed per mg.

TABLE 11. MINIMAL EFFECTIVE DOSES OF THYROTROPHIN, GROWTH HORMONE, AND ADRENOCORTICOTROPHIC HORMONE FOR CALORIGENESIS AND THYROID STIMULATION IN HYPOPHYSECTOMIZED RATS

Preparation	MED μg./da./14 da.			
	Metabolic rate	Thyroid weight	I^{131} uptake	Thyroid histology
TSH*	10	25	5	5
GH†	25	>100<500	>100<500	>100<500
ACTH‡	10	>50	>10<25	25

* Fels thyrotrophin.
† Ellis and Li growth hormone.
‡ Li α-corticotropin (administered in 5% beeswax peanut oil mixture).

of thyroid is not further increased. It is evident that the manner in which activity of the thyroid is expressed may influence the interpretation of the activity of the gland. With this caution in mind it is suggested that both TSH and GH are required to maintain a normal balance between the mass of body tissue utilizing the thyroid hormone and the mass of secreting thyroid tissue.[6]

Since the adrenal cortex has been shown to influence calorigenesis in the hypophysectomized rat the possibility exists that the calorigenic action of thyroxine or thyrotropin is partly mediated through stimulation of the adrenal and the increased release of adrenal cortical hormones. However, the adrenal cortex of the hypophysectomized rat showed little evidence of stimulation as judged by weight, morphology, or lipid pattern, after growth hormone was administered with either thyrotrophin or thyroxine. Hydrocortisone increased the survival rate when given with high levels of thyroxine and growth hormone though remarkably high metabolic rates were attained.

Other workers have also suggested that the anterior pituitary secretes more than one factor which is related to thyroid function (15, 16, 17). This factor (or factors) has not as yet been defined, and for this reason these preliminary experiments have been presented in the hope of stimulating further work on the elucidation of the balance of hormonal factors concerned in calorigenic homeostasis and normal thyroid function. Houssay has summarized the feeling of many investigators in this regard: ". . . on every problem of hormonal action, we must take into consideration the balance and interaction of the hormones. It is perhaps, one of the most important and difficult aspects of endocrinology." (18)

Acknowledgment

The authors wish to express particular thanks to Dr. Irving I. Geschwind and to Dr. Tetsuo Hayashida for their collaborative efforts in certain aspects of this work. The generosity of the contributors of hormonal preparations, acknowledged in the appendix, is much appreciated.

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APPENDIX

All rats were fed a standard laboratory diet consisting of 67.5% wheat, 15% casein, 7.5% skim milk powder, 6.75% hydrogenated vegetable oil, 1% fish oil, 0.75% NaCl, 1.5% $CaCO_3$, KI added (analysis 0.9–1.0 μg. iodine per gram of diet). They were kept at a constant temperature of $76 \pm 2°$ F. Following the surgical procedures the animals were injected intraperitoneally, for two consecutive days, with 1 cc. of 0.5% Terramycin in 5% glucose solution per 100 gm. body weight.

Hypophysectomy was performed through the parapharyngeal approach. In addition to the standard laboratory diet, fed dry, hypophysectomized and hypophysectomized-thyroidectomized rats received a wet mash of the same diet every afternoon. Rats were selected for experimentation on the basis of an immediate cessation of growth, impaired muscle tone, persistence of juvenile hair, and failure of vaginal membrane rupture. Completeness of operation was further confirmed at autopsy by examination under a binocular dissecting microscope of the sella turcica. The atrophic condition of the pituitary target organs offered additional confirmatory evidence of completeness of hypophysectomy.

Thyroidectomy was accomplished surgically, the completeness of operation being judged on the basis of body growth. In completely thyroidectomized male rats growth stasis occurred within 40 days at a maximum of 140 gm. Impaired muscle tone, persistence of juvenile hair, and low metabolic rate gave additional confirmatory evidence. The completeness of thyroid removal was checked routinely at the end of the experiment by the intraperitoneal administration of a tracer dose of I^{131} (1 μc.). Eighteen hours later the rats were sacrificed and the entire ventral neck region was removed, placed in a vial, and the I^{131} uptake was counted directly in a scintillation counter. The difference between radioactivity per minute and that in a comparable piece of leg musculature gave the counts per minute taken up by any thyroid remnant. The uptake was then expressed as the percentage of the total quantity of radioactivity administered. All rats in which tissue from the neck region incorporated more than 1% of the comparable region of the normal rat were considered incompletely thyroidectomized and rejected.

The hormonal preparations used and their standardizations are as follows:

Thyrotrophin was prepared from bovine pituitary glands according to the published method (2). Its potency was standardized in hypophysectomized rats by the histological response of the thyroids. The preparation was effective at the minimal total dose of 5 μg. given in 4 days. It was free of follicle stimulating hormone, lactogenic hormone, adrenocorticotrophic hormone and growth hormone at the doses employed. When administered for 14 days at doses of 500 μg. a day and above a small contamination with

interstitial cell stimulating hormone was apparent. A sensitive immunological assay for growth hormone contamination employing a potent and highly specific growth hormone antiserum from rabbits was carried out by Dr. Hayashida (3). On the basis of these tests it was estimated that the thyrotrophin contained less than 0.05% growth hormone. The hormone was administered by the intraperitoneal route in aqueous solution at pH 8.5. The same lot of thyrotrophin (G-94) was used in all experiments presented in this study.

The *growth hormone preparations* used are identified in the paper by lot numbers as follows:

L2722BS was prepared by C. H. Li according to the published method (4), VIII-34 was prepared by Stanley Ellis according to the published method (5), G-28 and G-43 were prepared by Grant Noda according to the method of Ellis, Noda, Simpson and Evans (5). The potency of these preparations was standardized in hypophysectomized rats by the response of the proximal epiphyseal cartilage plate of the tibia. All preparations were effective at a minimal total dose of 30 μg. given in 4 days. They were free of follicle stimulating hormone, lactogenic hormone and adrenocorticotrophic hormone at the doses employed. A positive reaction for thyrotrophin and interstitial cell stimulating hormone at total doses of 1–2 mg. given in 4 days were observed in hypophysectomized rats. The preparations were administered by the intraperitoneal route in aqueous solution at pH 8.5.

Stable suspensions of Merck *hydrocortisone acetate* were diluted with 0.9% NaCl and injected subcutaneously in a volume of 0.1 cc.

Sodium *l*-thyroxine was dissolved in alkaline 0.9% NaCl solution and injected intraperitoneally.

HORMONAL EFFECTS ON PHOSPHORYLASE ACTIVITY IN THE RAT UTERUS[1]

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ABSTRACT

Hormonally induced changes in total phosphorylase *t* and active phosphorylase *a* concentrations were determined in the uteri of spayed rats given estradiol and the ratios *a*/*t* calculated. A single injection of 50 μg. estradiol in rats spayed 6–8 days increases phosphorylase *a* concentration and the ratio *a*/*t* at least 2 fold in 48 hours without altering the level of phosphorylase *t*. The amount of phosphorylase *a* per whole uterus increases by 6 hours after estradiol, phosphorylase *t* by 24 hours, indicating 2 phases of the estrogen effect. Phosphorylase *a* is high at normal estrus, low during diestrum. Testosterone acts similar to estradiol but is less effective and progesterone has no effect.

Large doses of adrenaline, nor-adrenaline and posterior pituitary extract given intraperitioneally to estrogen-primed rats, markedly lowers phosphorylase *a* within 1 hour, but not phosphorylase *t*. Histamine lowers both slightly without altering the ratio. These changes in phosphorylase *a* parallel similar changes in uterine glycogen, previously reported.

ESTROGENS are highly specific hormones for increasing glycogen stores in the rat uterus (1, 2, 3) and intraperitoneal injections of posterior pituitary extract, oxytocin, and the adrenal medullary hormones rapidly deplete the glycogen (4). Since phosphorylase is an important enzyme in glycogen metabolism of skeletal muscle and liver (5, 6), the following study was made on uterine phosphorylase in an attempt to correlate possible changes in enzyme activity with changes in glycogen, induced by these hormones.

MATERIALS AND METHODS

Adult Long-Evans rats (220–250 gm.) were used in all experiments except one. The standard procedure was to ovariectomize the rats 6–8 days before hormone treatment. Estradiol[2] (50 μg. in 0.1 cc. of peanut oil) was given in a single injection subcutaneously 48 hours before autopsy. In some experiments, testosterone propionate (2 mg daily) and progesterone (3 mg. daily) in oil were given subcutaneously, 3 days before autopsy. The following were given intraperitoneally in 1 cc of water to estrogen-primed rats (as above), 1 hour before autospy: Posterior pituitary extract (Jensen-Salsbery Lab., 1 U.S.P.-U.), *l*-adrenaline-HCL (Parke, Davis Co., 100 μg.); *d*, *l*-noradrenaline-HCl

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[2] The Schering Corporation, Bloomfield, N.J. generously furnished the steroid hormones used in these experiments.

(Winthrop-Stearns, 500 μg.); histamine-HCl (2mg.). In one experiment, adrenaline (250 μg.) was given subcutaneously 3 hours before autospy.

The uteri were removed while the rats were under nembutal anesthesia, quickly trimmed of extraneous connective tissue and the cervix, emptied of fluid by blotting and frozen between 2 blocks of dry ice. The frozen tissue was wrapped in parafilm and stored until used at −20 C without loss of weight or enzyme activity. To secure sufficient tissue for a complete assay, pooling of 2 or more uteri was usually necessary.

Sutherland's method for phosphorylase assay as previously described (7) was employed, only using the rate of liberation of inorganic P from glucose-1-PO_4 as a measure of enzyme activity. Elimination of the glycogen primer, in a test reaction, prevented the formation of inorganic P which indicated no other enzymatic source of P. Forty mg. of tissue homogenate per cc. were required for each tube. Since glycogen is practically all localized in the myometrium (2,8) and if it may be presumed that phosphorylase is in the same cells with the glycogen, it is obvious that the endometrial contribution is one factor responsible for the relatively large amount of uterine tissue required for each assay. Both active phosphorylase *a* and total phosphorylase *t* (requiring adenylic acid) were measured as μg P liberated from glucose-1-PO_4 by 40 mg. uterine tissue in 10 minutes at 37.5 C. Using the μg. P as arbitrary units, the ratios of $a/t \times 100$ were calculated. It is essential to assay tissue from control and experimental animals at the same time in any one experiment to circumvent an unaccounted variation between different groups of rats.

RESULTS

In a preliminary experiment, the rats were spayed one month before they received a single injection of 50 μg. of estradiol. The results on the first line of Table 1 shows that in 48 hours, phosphorylase *a* concentration is more than doubled, that the total phosphorylase *t* concentration is unchanged and that the increased ratio a/t is a reflection of the effect on only

TABLE 1. HORMONAL EFFECTS ON PHOSPHORYLASE ACTIVITY IN THE RAT UTERUS

Experiment[1]	μg. P ±S.E.		Ratio a/t ×100	Control[1]	μg. P ±S.E.		Ratio a/t ×100
	P-lase a	P-lase t			P-lase a	P-lase t	
Estradiol (4)	*41.8* ±3.2	118.0 ±6.8	*35.4* ±1.8	no hormone (4)	14.5 ±2.9	129.5 ±5.2	11.3 ±2.0
Normal estrus (5)	*39.4* ±2.8	95.2 ±9.1	*42.7* ±4.3	Normal diestrum (5)	19.0 ±2.3	87.8 ±9.0	21.6 ±1.4
Testosterone (8)	*33.8* ±2.3	131.0 ±4.1	*25.6* ±1.4	no hormone (7)	22.4 ±1.2	128.3 ±6.9	17.6 ±0.7
Estradiol + Post. Pit. ip. (4)	*20.5* ±1.5	111.0 ±2.6	*18.4* ±1.1	Estradiol (4)	41.3 ±1.6	118.8 ±1.3	34.7 ±1.1
Estradiol + Adrenaline, ip. (5)	*16.0* ±1.5	114.6 ±3.7	*13.9* ±1.2	Estradiol (5)	30.2 ±1.2	113.4 ±6.6	26.8 ±1.4
Estradiol + Adrenaline, sc. (4)	53.8 ±2.8	104.8 ±3.4	51.3 ±1.3	Estradiol (4)	50.3 ±1.5	96.0 ±4.4	52.5 ±1.3
Estradiol + Histamine, ip. (7)	*51.6* ±2.8	*85.0* ±2.5	60.6 ±2.6	Estradiol (5)	63.0 ±2.2	107.0 ±4.0	59.1 ±4.5

[1] All rats spayed except in 2nd experiment. ( ) refers to number of assays; ip. = intraperitoneal; sc. = subcutaneous; details given in text. Data in italics significant, P = .01 or less.

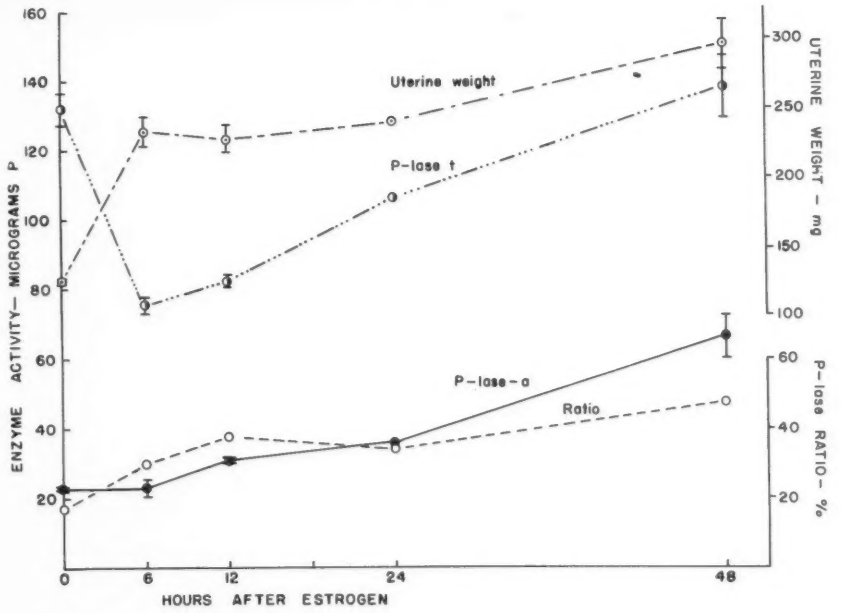

FIG. 1. Changes in phosphorylase *a* and *t* concentration in rat uteri at intervals after a single injection of estradiol, uterine weights, with standard errors. Enzyme activity is expressed as μg. P liberated from glucose-1-PO_4 by 40 mg. of tissue in 10 minutes at 37.5° C. Ratio—*a*/*t*. Details in text.

active phosphorylase. Subsequently, rats spayed for only 6–8 days were found equally satisfactory to demonstrate this response.

To determine the rate at which estrogen increases the enzyme, 48 rats (purchased from a commercial breeder and each weighing 180–200 gm) were spayed, 7 days later injected with estradiol and autopsied at intervals. The number of assays made were 4, 6, 6, 2, 4 at intervals of 0, 6, 12, 24, 48 hours respectively. The results, together with the average uterine weights are shown in Figure 1. Six hours after injection, phosphorylase *a* level was unchanged, the total phosphorylase decreased by 43% and the activity ratio increased. The uterine weight increased markedly as expected. By 12 hours, both phosphorylase *a* and *t* concentration began to increase and continued up to 48 hours. By now, phosphorylase *t* returned to the initial level, but phosphorylase *a*, as well as the activity ratio had nearly trebled. The uterine weight during this time increased more slowly up to 48 hours.

The total amount of enzyme activity per uterus was calculated for each interval and the results are presented in Figure 2. Now it is observed that phosphorylase *a* activity in the whole uterus increased by 88% in the first 6 hours while phosphorylase *t* remained practically unchanged. By 24

hours, a substantial increase in phosphorylase *t* had occurred and by 48 hours, both phosphorylase *a* and *t* were considerably higher as the uterus increased further in size.

The question concerning the effect of estrogen treatment beyond 48 hours was considered in the following experiment. A group of 32 Long-Evans rats were injected once with 50 μg. estradiol, 8 were killed at 48 hours, the remainder received 25 μg. daily for 3 more days. Rats were autopsied on days 3, 4, 5 after the initial injection, making 4 assays at each interval. The average enzyme level, as μg. P on days 2–5 are in order:

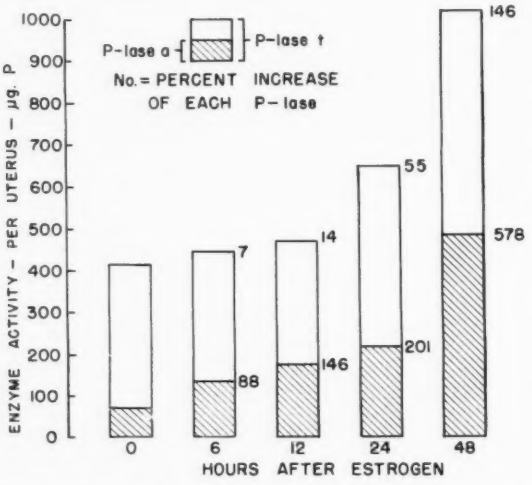

FIG. 2. Calculated phosphorylase *a* and *t* activity, expressed as μg. P that would be liberated by a single uterus, at each interval after estrogen.

phosphorylase *a* 50, 62, 71, 61; phosphorylase *t*, 96, 109, 120, 117; the respective ratios, 53, 57, 59, 53. Unfortunately, no uninjected controls were assayed in this experiment but it appears, nevertheless, that only a relatively small increase in enzyme activity beyond 48 hours occurs with prolonged estrogen treatment. The ratios were not statistically different.

To determine if changes in enzymes activity could be detected in the normal sex cycle, rats were selected in "estrus" (vaginal cells nucleated or cornified) and in "diestrum" (cell population only leucocytes). The results in Table 1 indicate phosphorylase *a* is high during estrus and low during diestrum while total phosphorylase remains unchanged.

The effects of testosterone on uterine phosphorylase were also determined. Data in Table 1 show that the effects are like those of estradiol but definitely much weaker. The activity ratios are increased by only 45.5% as compared to 200–300% increase obtained with estradiol.

Progesterone was also tested although no effect was expected since it does not increase uterine glycogen (3, 8). Three assays after progesterone treatment showed an average phosphorylase activity ratio of 28.8, while concurrently, a spayed control was 24.8 and an estradiol-treated control was 60.0. Progesterone alone in spayed rats was ineffective.

Since intraperitoneal injections of large doses of posterior pituitary extract, adrenaline and noradrenaline, will decrease the uterine glycogen in 1 hour in an estrogen-primed rat (4) assays were made on the enzyme under similar conditions. One hour after intraperitoneal injections of posterior pituitary extract or adrenaline, phosphorylase *a* and the activity ratios decreased to almost one-half the control levels, while the total phosphorylase was unchanged (Table 1). Similar results were obtained with noradrenaline, the average activity ratios of 2 assays was 28.8 as compared to 63.8, the average of 2 controls. However, larger doses of adrenaline administered subcutaneously were unable to lower phosphorylase in 3 hours.

Based on the possibility that histamine released by the large doses of adrenaline might be the cause of the decrease in phosphorylase, 2 mg doses of histamine were injected intraperitoneally into estrogen-primed rats. A small but statistically significant decrease in phosphorylase *a* and *t* occurred but the ratios were unchanged (Table 1).

DISCUSSION

All of the hormonally induced changes in rat uterine phosphorylase activity can be correlated with changes in uterine glycogen concentration previously observed in this laboratory (3, 4) or noted elsewhere (1, 2). Since glycogen is practically all in the myometrium (1, 2, 8), this may be considered as presumptive evidence that the enzyme is similarly located. The inclusion of the endometrium would not necessarily negate relative changes in the enzyme (or glycogen) but only the absolute values.

The observed differences in the concentration of phosphorylase *a* and *t* between successive experiments where rats received similar treatment, cannot be explained at present. These differences in no way invalidate the results and the variation has been circumvented by assaying appropriate control animals concomitantly in each experiment, using the same homogenizer and the same solutions.

Analysis of the data in conjunction with previous studies on uterine changes induced by estrogen indicate 2 phases in this hormone's effect on phosphorylase. The first, temporally, is an increase in phosphorylase *a*, probably by a conversion of the inactive phosphorylase *b* to *a* according to the scheme proposed for this enzyme system (9, 10, 11). It is upon this conversion mechanism that future analysis of the effect of estrogen must be sought. At the end of 6 hours after an estrogen injection, phosphorylase *a* appears unchanged and phosphorylase *t* markedly decreases (Fig. 1). However, a doubling of uterine weight also occurs, due almost entirely to the

uptake of water (1, 2, 12). Thus a "dilution effect" explains the apparent decrease in phosphorylase *t* but phosphorylase *a* must have increased to maintain it's initial concentration in the enlarged uterus. Calculation of the enzyme changes based on the entire uterus (Fig. 2) clearly shows that phosphorylase *a* increases during the first 6 hours and that the amount of phosphorylase *t* remains practically unchanged.

The second phase of the estrogen effect on the enzyme is observed 24 hours after injection as a substantial increase in phosphorylase *t* concentration. At this time, an increase in dry weight, and a decrease in the percentage of water occurs in the uterus (1, 12). Calculated on the basis of the entire uterine weight, phosphorylase *t* has increased 55% and at 48 hours, 146%. Meanwhile, more phosphroylase *a* is formed so that at 48 hours, the ratio *a*/*t* is more than doubled as the concentration of phosphorylase *t* is restored to the initial level. Correlated with these changes, it has been shown that a single injection of 100 R.U. of estradiol dipropionate into a spayed rat produces the highest concentration of glycogen in 46 hours (1). Continued injections of estrogen up to 5 days produces only a slightly higher increase in the enzyme concentration.

In the normal estrus cycle, phosphorylase *a* was high during estrus, and low in the diestrus period, corresponding to the high and low levels of glycogen (1). Phosphorylase *t* was unchanged but it is possible that analyses made at smaller intervals during the cycle might reveal a change here also.

Although testosterone induces growth and glycogen deposition in the rat uterus, similar to an estrogen effect (3), it is considered a weak stimulator. The changes in phosphorylase *a* following testosterone treatment are also small compared to those produced with estradiol. Progesterone fails to stimulate phosphorylase or glycogen deposition (3).

A single intraperitoneal injection of either posterior pituitary extract, adrenaline or noradrenaline into an estrogen-primed rat lowers phosphorylase *a* within 1 hour, without affecting phosphorylase *t*. Under similar conditions, glycogen concentration also diminishes (4). Subcutaneous injection of adrenaline is without effect in both instances. It is not known why large doses of these hormones, given intraperitoneally, affect the metabolic machinery of uterine muscle when only small amounts modify contractile behavior. These hormones produce opposite effects on uterine motility yet always decrease enzyme activity.

A recent report appeared in which only total phosphorylase (adenylic acid present) per unit weight of human myometrium was determined at different reproductive periods (13). The enzyme concentration was lowest at the menopause, higher during the menstrual cycle, progressively became still higher during the course of pregnancy and decreased after parturition. The activity ratio was stated to be constant, about 65%, at all stages and myometrial glycogen concentration and enzyme activity increased together.

It is undoubtedly premature to make generalized statements in regard to phosphorylase activity and changes in glycogen levels within all pertinent tissues. At equilibrium, the enzyme reaction *in vitro* is heavily in the direction of glycogen synthesis which correlates nicely with the results found in the rat myometrium under hormonal influence. However, in skeletal muscles, adrenaline *increases* phosphorylase *a* and lowers glycogen (5, 6, 7), which suggests independent factors are affecting the equilibrium of the reaction within the cells, or are affecting some other stage in glycogen metabolism.

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RESPONSE OF VITAMIN B_6-DEFICIENT RATS TO HYPOPHYSEAL FOLLICLE-STIMULATING AND INTERSTITIAL-CELL-STIMULATING HORMONES[1]

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ABSTRACT

The response of vitamin B_6-deficient rats to varying amounts of FSH and ICSH was determined and compared with that of hypophysectomized adult rats. The adult hypophysectomized rats required twice the amount of FSH for stimulation of growth of ovarian follicles and for increased uterine weights as was required by the immature hypophysectomized rats used to standardize the hormone preparation. Eight times the amount of FSH effective in hypophysectomized adult rats was required to stimulate follicular growth in the ovaries of vitamin B_6-deficient animals. Estrin production in B_6-deficient rats, as judged by increased uterine weights, required two to four times as much FSH as in hypophysectomized animals of the same body weight. Repair of the atrophic interstitial tissue was obtained in B_6-deficient animals when approximately twice the dose level of ICSH effective in either immature or adult hypophysectomized rats was given.

PREVIOUS studies (1, 2) have shown that fetal death and resorption consistently occurred in adult rats subjected to a vitamin B_6-deficient diet containing the antimetabolite, deoxypyridoxine. The reproductive failure was apparently due to lack of the ovarian hormones as pregnancy could be maintained in 90% of such B_6-deficient rats by administration of estrone and progesterone (3) at the same dosage levels shown to be effective in rats oophorectomized after breeding (4). Lyons, Simpson and Evans (5) further demonstrated that in hypophysectomized rats the three hypophyseal gonadotrophins could be substituted for the synthetic ovarian hormones to maintain pregnancy. When these hypophyseal hormones were

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used in B_6-deficient rats, only 25% had shown living young at autopsy (6). Furthermore, a FSH-ICSH preparation given with progesterone at the dosage levels successfully used in hypophysectomized rats (7) did not maintain pregnancy in B_6-deficient rats to any significant extent.

These results suggested the possibility of decreased ovarian sensitivity of vitamin B_6-deficient rats to one or more of the hypophyseal gonadotrophins. To test this hypothesis, virgin B_6-deficient female rats were injected with varying levels of FSH and of ICSH and the response to each hormone compared with that obtained in adult hypophysectomized rats of similar ages and body weights.[3] The FSH and ICSH preparations were standardized in immature hypophysectomized female rats and the FSH preparation was also tested in normal immature rats.

EXPERIMENTAL METHODS

Virgin rats of the Long-Evans strain were placed on the vitamin B_6-deficient diet[4] when 80 to 85 days of age. Vaginal smears were examined daily and the rats were weighed and checked every 5 days for acrodynia. After 60 days on the diet, those B_6-deficient animals showing definite signs of deficiency (e.g., cessation of growth, progressive acrodynia, and loss of the estrous cycle) were distributed into equivalent groups for hormone injection on the basis of length of anestrus and body weight. The animals had been anestrous for an average of 40 days and showed an average loss in body weight of 9%.

Adult female rats maintained on a stock diet[5] of natural foodstuffs were hypophysectomized at 80 to 84 days of age and injections started 19–20 days post-operative; these animals were similar in body weight to the B_6-deficient rats when injections were given. The immature hypophysectomized female rats used for standardization of the hormones were operated when 26–28 days of age and tested 12–18 days later. The normal immature rats were 24 days of age at the beginning of the injection period.

The FSH[6] and ICSH[7] preparations were given by subcutaneous injections once daily

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[3] Preliminary reports of this study have been made before the American Institute of Nutrition (8, 9).

[4] The vitamin B_6-deficient diet was the same as that used in previous studies (e.g., 3, 6). It consisted of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco or Primex), 4% salts No. 4, and 0.5 mg. % deoxypyridoxine, the vitamin B_6 antimetabolite. Crystalline vitamins per kilogram of diet were: 300 μg. *d*-biotin, 5 mg. 2-methyl-1,4-naphthoquinone, 5 mg. thiamine HCl, 5.5 mg. pteroylglutamic acid, 10 mg. riboflavin, 10 mg. *p*-aminobenzoic acid, 20 mg. niacin, 50 mg *d*-calcium pantothenate, 400 mg. inositol, and 1.0 gm. choline chloride. Each rat received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 chick units vitamin D, 6 mg. synthetic *dl*-alpha-tocopherol, and 650 mg. corn oil (Mazola).

[5] The stock diet (14) was composed of 68.5% ground whole wheat, 10% fish meal, 10% alfalfa leaf meal, 5% casein, 5% fish oil (Sardilene), and 1.5% iodized NaCl. Lettuce was given *ad libitum* twice weekly. After hypophysectomy, the animals received in addition wet stock diet I composed of 67.5% ground whole wheat, 15% casein, 7.5% skim milk powder, 6.75% hydrogenated vegetable oil, 1.5% $CaCO_3$, 1% fish oil (Sardilene), and 0.75% iodized NaCl.

[6] FSH was prepared from sheep pituitaries by ammonium sulfate fractionation. The MED or one Rat Unit (R.U.) (10), as determined in immature hypophysectomized rats was 40 μg. (30–50 μg.). The FSH preparation is the same as that used in previous

for 4 days and the rats were autopsied 24 hours after the last injection. Ovaries and uteri were weighed at autopsy. The ovaries were fixed in Bouin's fluid, serially sectioned, and stained with hematoxylin and phloxine for histological study. The levels of FSH tested ranged from 1 to 32 Rat Units (R.U.), and the levels of ICSH tested varied from ½ to 10 R.U. Three to five animals were included in each test group and each dose level was repeated at least once. The minimal effective dose (MED) for follicular growth and for interstitial tissue repair and the response to multiples of the MED were determined for the normal immature,[8] the immature and adult hypophysectomized, and B_6-deficient rats. The end point used to determine the MED of FSH was an increase in number and size of ovarian follicles when compared with the respective uninjected controls. An additional criterion for ovarian response was a significant increase in uterine weight, indicating estrin production. The end point used to determine the MED for ICSH was a histologically detectable response, designated as partial repair, of the interstitial tissue, i.e., resumption of the normal nuclear pattern in these cells with some increase in cytoplasm.

RESULTS

The response to varying levels of *FSH* is shown in Table 1. Injection of one R.U. of FSH in *immature hypophysectomized rats*, 2–3 weeks post-operative, resulted in stimulation of follicular growth, i.e., a change in size of follicles from small to small-medium with beginning antrum formation. Increased uterine weights, indicating estrin production, occurred at 4 R.U. Repair of the "deficient" interstitial tissue was not observed at any dosage level given.

Normal immature rats at autopsy (28 days of age) had ovaries containing small-medium and a few medium follicles. In these animals a dose of 2 R.U. of FSH was required for stimulation of follicular growth, and resulted in a significant increase in number of follicles (shown by actual follicle counts). Uterine weights increased significantly when 8 R.U. were injected. Thus, twice as much FSH was required by the normal immature rat for stimulation of both follicular and uterine growth as was necessary for the immature hypophysectomized rat.

Adult hypophysectomized rats, approximately 3 weeks post-operative, had ovaries containing small follicles, atrophic interstitial tissue, and residual corpora lutea. A dose of 2 R.U. of FSH was required for follicular

studies (5, 6) in which it was referred to as an FSH-ICSH combination inasmuch as it contains approximately 5–10% ICSH; however, ICSH activity, as shown by repair of "deficient" interstitial tissue, was not evident until 40 R.U. were given subcutaneously.

[7] ICSH was prepared from beef pituitary by ammonium sulfate fractionation. Two ICSH preparations were used in this study. The MED or one R.U. (10) as determined in the immature hypophysectomized rat for one preparation was 50 μg. when given by I.P. injection or 200–250 μg. when injected subcutaneously. The MED for the second preparation was 15 μg. by I.P. injection and 60 μg. by subcutaneous injection. The data obtained by these two ICSH preparations were in agreement and have therefore been combined in the tables.

[8] The ICSH preparations at the dosages used in the present study had no detectable effects on the ovaries of normal immature rats.

stimulation, i.e., a change from small to small-medium follicles with beginning antrum formation. Increased uterine weights were first observed at 8 R.U. Thus, the amount of FSH required for minimal follicular stimulation and uterine growth in these animals hypophysectomized after maturity was double that required by animals hypophysectomized during immaturity, i.e., 26–28 days of age.

TABLE 1. RESPONSE OF FEMALE RATS TO HYPOPHYSEAL FOLLICLE-STIMULATING HORMONE

Total dose R.U.	No. of rats	Ovaries			Uterus[3] weight, mg.
		Weight,[1] mg.	Histology[2]		
			Immature hypophysectomized rats		
0	19	7 ± 1	s F	defic. IT	14 ± 1
1	7	12 ± 1	*sm F*	defic. IT	14 ± 1
2	7	15 ± 1	sm, m F	defic. IT	15 ± 1
4	8	27 ± 4	m, ml, few l F	defic. IT	44 ± 8
8	9	29 ± 5	ml, l F	defic. IT	79 ± 7
16	7	27 ± 5	ml, l F	defic. IT	83 ± 7
			Immature normal rats		
0	14	16 ± 1	sm, few m F		27 ± 2
1	5	18 ± 5	sm, few m F		26 ± 2
2	9	18 ± 1	*incr. s, sm, and m F*		29 ± 2
4	14	22 ± 1	incr. s, sm, and m F		31 ± 2
8	9	25 ± 1	m, few ml F		44 ± 4
16	9	38 ± 6	m, ml F, few CL		81 ± 12
			Adult hypophysectomized rats		
0	9	35 ± 3	s F	atrophic IT	78 ± 4
1	10	33 ± 2	s F	atrophic IT	65 ± 3
2	9	35 ± 1	*sm F*	atrophic IT	78 ± 3
4	10	41 ± 3	sm, m F	atrophic IT	86 ± 6
8	10	57 ± 3	m, ml, few l F	atrophic IT	148 ± 17
16	10	68 ± 5	many m, ml, l F	atrophic IT	223 ± 10
			B_6-deficient rats		
0	24	32 ± 2	m, few ml F	atrophic IT	99 ± 4
8	13	35 ± 3	m, few ml F	atrophic IT	97 ± 7
16	11	41 ± 4	*m, ml, few l F*	atrophic IT	119 ± 8
32	8	51 ± 5	many m, ml, l F some lut, few CL	atrophic IT	250 ± 34

[1] Mean ± standard error of the mean.

[2] Abbreviations: F = follicles, lut = luteinized, CL = corpora lutea, IT = interstitial tissue, p. repair = partial repair; s = small, m = medium, l = large refer to the size of the follicles which were measured with a calibrated eyepiece micrometer. The standards are those used by Wooten *et al.* (10), s = up to 375 μ, sm = 450–500 μ, m = 550–650 μ, ml = 700–750 μ, l = 800–1000 μ.

[3] Uterus was dissected free of the oviducts and drained of fluid before weighing.

Vitamin B_6-deficient animals after two months of deficiency had ovaries containing medium-sized follicles, atrophic interstitial tissue and residual corpora lutea as previously reported (10). Occasionally a few medium-large follicles were observed. At a dose of 8 R.U. of FSH there was no evidence of stimulation of the ovaries, uterus or vagina. With 16 R.U. definite signs of follicular growth occurred; medium to medium-large follicles were increased in number and a few large follicles were present. At 32 R.U. the

follicles were further increased in number and some were luteinized; newly formed corpora lutea were present in the ovaries of a few animals. At this dose there was no detectable change in interstitial tissue. Average uterine weights were increased at this level (32 R.U.) and estrous vaginal smears were observed before autopsy.

The response to graded levels of *ICSH* is shown in Table 2. Injection of one R.U. of ICSH resulted in partial repair of interstitial tissue in both immature and adult hypophysectomized rats. In the vitamin B_6-deficient animals, partial repair of the interstitial tissue was not obtained until 2

TABLE 2. RESPONSE OF FEMALE RATS TO HYPOPHYSEAL INTERSTITIAL-CELL-STIMULATING HORMONE[1]

Total dose R.U.	No. of rats	Ovaries			Uterus weight, mg.
		Weight, mg.	Histology		
			Immature hypophysectomized rats		
0	29	7 ± 1	s F	defic. IT	15 ± 1
½	13	8 ± 1	s F	defic. IT	14 ± 1
1	26	8 ± 1	s F	*p. repair*	14 ± 1
2	11	8 ± 1	s F	p. repair-repair	15 ± 1
4–5	7	11 ± 1	s F	repair	17 ± 1
8–10	6	9 ± 1	s F	repair	16 ± 1
			Adult hypophysectomized rats		
0	16	31 ± 2	s F	atrophic IT	72 ± 4
½	7	29 ± 1	s F	atrophic IT	84 ± 5
1	15	29 ± 2	s F	*p. repair IT*	88 ± 5
2	16	32 ± 2	s F	*p. repair IT*	87 ± 3
4	9	31 ± 2	s F	*p. repair IT*	89 ± 7
			B_6-deficient rats		
0	11	30 ± 2	m, few ml F	atrophic IT	99 ± 6
1	7	33 ± 3	m, few ml F	atrophic IT	92 ± 8
2	8	37 ± 3	m, few ml F	*p. repair IT*	95 ± 8
4	7	35 ± 3	some lut.	*p. repair IT*	102 ± 13
8–10	8	39 ± 4	some lut.	*p. repair IT*	102 ± 8

[1] See Table 1 for abbreviations.

R.U. were given. Further increases in dose resulted in somewhat larger interstitial cells and in luteinization of some follicles.

DISCUSSION

The data summarized in Table 3 demonstrate that B_6-deficient rats as compared with adult hypophysectomized animals showed a markedly decreased response to FSH with a slight decrease in response to ICSH. The amount of FSH needed for initiating follicular growth in the ovaries of of B_6-deficient rats was 8 times that required by hypophysectomized animals of the same body weight. The FSH preparation was likewise less effective in stimulating the production of estrin, as judged by uterine weights the MED for the B_6-deficient rats being two- to four-fold the dose required

for hypophysectomized adult rats. The MED of ICSH for repair of interstitial tissue in B_6-deficient rats was approximately twice that required for hypophysectomized animals of similar body weights.

The decreased response of vitamin B_6-deficient rats to injected FSH (and to ICSH) offers a possible explanation for the failure of these hypophyseal gonadotrophins when given with either progesterone or lactogenic hormone to maintain pregnancy in B_6-deficient animals (6). The dosage levels of gonadotrophins used in the latter study were those found effective in the adult rat hypophysectomized after breeding (5).

The decreased response to injected FSH (and to ICSH) in vitamin B_6-deficient animals may be due to (a) decreased sensitivity of the ovary which would explain the increased gonadotrophic potency of the pituitary

TABLE 3. COMPARISON OF THE MED'S FOR THE SPECIFIC EFFECTS OF HYPOPHYSEAL FOLLICLE-STIMULATING AND INTERSTITIAL-CELL STIMULATING HORMONES IN FEMALE RATS[1]

Test Animal	Autopsy Body Weight, gms.	Ovarian Histology of uninjected animals	Minimal effective dose for:	
			FSH Follicular stimulation	*ICSH* Repair of interstitial tissue
Immature hypophysectomized	65	s F, deficient IT	1 R.U.	1 R.U.
Immature Normal	65	sm, few m F	2 R.U.	
Adult Hypophysectomized	175	s F, atrophic IT, CL	2 R.U.	1 R.U.
Adult B_6-Deficient	175	m, few ml F atrophic IT, CL	16 R.U.	2 R.U.

[1] See Table 1 for abbreviations.

resulting from B_6-deficiency (11), (b) absence of synergistic factors or presence of inhibitory factors, or (c) increased destruction or inactivation by body tissues or increased rate of excretion. At the present time little information is available as to which factors are concerned in B_6-deficient animals. It may be noted that of the three criteria used, one, namely, increase in uterine weight, involves not only the ovarian response to gonadotrophins but also the uterine response to estrogen. The response of B_6-deficient rats to estrogen has been assumed to be normal, inasmuch as pregnancy can be maintained in such rats by injection of estrone and progesterone at the same dosage levels effective in the hypophysectomized-ovariectomized rat (3). However, the sensitivity of B_6-deficient rats to estrogen alone is being investigated.

The B_6-deficient rats were similar to but not identical with the adult hypophysectomized animals in the degree of gonadal atrophy. Functionally, ovaries of both types of rats were subnormal and did not produce sufficient quantities of either estrin or progestin to maintain pregnancy. Morphologically, the atrophic interstitial tissue and residual corpora lutea

were similar in both animals. However, there was considerably more follicular development in ovaries of B_6-deficient rats than in those of adult hypophysectomized animals. The degree of follicular development in the majority of B_6-deficient rats was very close to that observed in normal immature rats, which required the same levels of FSH as the adult hypophysectomized animals. However, ovaries of normal immature rats did not contain either corpora lutea or atrophic interstitial tissue. Attempts have been made to hypophysectomize B_6-deficient animals during the latter part of the deficiency period but the survival rate has been very low, less than 20%.

Few studies have been made on the response to purified hypophyseal gonadotrophins of rats deficient or lacking in other specific dietary essentials. The only comparable study is that of Srebnik *et al.* (12) on the response of rats to FSH in the absence of dietary protein. After 5 weeks on a protein-free diet, adult female rats with a loss in body weight of 30–35% were found to be as sensitive to FSH as the adult hypophysectomized rats reported in the present study, i.e., 2 R.U. of the same FSH preparation resulted in minimal follicular stimulation in the ovaries of either protein-deficient or hypophysectomized adult animals. Likewise, 8 R.U. of FSH resulted in increased uterine weights in both types of experimental animals. These results suggest that the decreased response to FSH observed in B_6-deficient rats was not due to the comparatively mild degree of inanition observed in this deficiency or to a relative protein deficiency induced by decreased food intake.

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SEPARATION OF LUTEINIZING HORMONE FRACTIONS FROM SHEEP PITUITARY GLANDS BY USE OF ION EXCHANGE RESINS[1]

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ABSTRACT

The effect of pH on the adsorption of LH and FSH from an aqueous extract of sheep pituitary glands by anion exchange resin XE-59 was studied and it was found that LH is selectively adsorbed at pH 8.5 and 9.0. Assays of these fractions at a dose of 0.5 gm. eq. in intact immature and hypophysectomized male rats produced ventral prostate weights approximately two and fourfold greater than control values. The FSH contamination in these fractions was low as shown by administration of high doses to intact and hypophysectomized female rats.

The LH fractions obtained with anion exchange resin XE-59 were further purified by fractionation on columns of cation exchange resin XE-97. The LH was eluted from the columns in two peaks, the first containing the major part of the activity whereas the second contained only a minor part. The fractions comprising the major peak of activity were combined and assayed in hypophysectomized male rats. The ventral prostates were increased threefold. A similar response was observed with the seminal vesicles whereas the effect on the testes was small. Histological study of these testes showed that the effect of LH was restricted to the interstitial tissue.

Only a very small amount of FSH was present in the purified fractions as shown by administering 5.0 gram equivalents to hypophysectomized female rats. The average increase in the weight of the ovaries was 3 mg. Histological study of these ovaries showed the repair of the interstitial tissue. The LH augmented the action of FSH on the ovaries and induced the formation of corpora lutea.

THE first preparation of a luteinizing hormone fraction was reported by Fevold, Hisaw and Leonard (1). Subsequently, other methods were developed for obtaining luteinizing preparations of varying degrees of biological and chemical purity (2–16). Classical procedures for fractionating proteins were used to obtain these preparations. The results of experiments presented in this report show that a part of the luteinizing hormone activity is selectively adsorbed from an aqueous extract of sheep pituitary tissue by an anion exchange resin and that purification is effected by use of a cation exchange resin.

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MATERIALS AND METHODS

Acetone dried sheep pituitary glands were used for the preparation of the luteinizing hormone (LH) fractions. The ion exchange resins used were XE-59(IR-4B), a weak anion exchange resin, and XE-97(IRC-50), a weak cation exchange resin, obtained from Rohm & Haas Company, Philadelphia, Pennsylvania.

Extraction. Samples of the dry sheep pituitary glands ranging from 25 to 100 gm. were suspended in distilled water in the ratio of 15 cc. per gm. of dry tissue and adjusted to pH 9.0 to 9.2 by the addition of 4*M* NaOH during stirring. This mixture was shaken for three hours at room temperature and centrifuged. The pituitary residue was extracted twice more at pH 9.0 with 10 cc. and 5 cc. of water per gm. of original dry glands by shaking for 3 and 2 hours, respectively. The three extracts were centrifuged separately a second time at 2500 rpm for 30 minutes, and the sediment which contained some gonadotrophic activity was discarded. The extracts were combined and adjusted to pH 8.5.

Preparation of anion exchange resin XE-59. Anion exchange resin XE-59, in lots of 100 gm., was suspended in 4 liters of distilled water, stirred into a homogeneous suspension and allowed to settle. The water was decanted to remove the "fines." This step was repeated twice after which the resin was suspended in 1.5 liters of distilled water and stirred into a uniform suspension. Three 1 cc. samples of the suspension were dried at 100° C and weighed to determine the total weight of the resin.

The resin was cycled through base, acid and base. It was treated first with 25 cc. of 1.25*M* NaOH per gm. of resin, stirred for 1.5 hours and diluted 4:1 with distilled water. The suspension was filtered on a large Buchner funnel using No. 202 filter paper (H. Reeve Angel and Company, New York) and it was washed by the continuous addition of distilled water until the resin flocculated when suspended in water. It was washed 3 more times by suspending each time in 3 liters of water, stirring and decanting after the resin had settled.

The anion resin was next stirred with 25 cc. of 1.25*M* HCl acid per gm. XE-59 and washed as described above until the filtrate did not turn blue litmus paper red. Finally, the resin was treated again with NaOH as already described. The concentration of the suspended resin was determined and the pH was adjusted with HCl to 8.5 for the adsorption. It should be pointed out that the most critical step of the procedure is the adjustment of the resin to the desired pH. Equilibrium was attained slowly which made it necessary to adjust the resin the day before it was used to insure equilibrium at pH 8.5. The pH during this period was checked frequently and the proper adjustments were made.

Selective adsorption of luteinizing activity on anion resin XE-59. Prior to adsorption, the pH of the resin and the pituitary extract was adjusted, if required, to 8.5. The resin and extract were combined in the ratio of 700 mg. of XE-59 per gram equivalent (gm. eq.) of extract. In preparing 100 gm. eq. of LH, 70 gm. of the resin was added to approximately 2,700 cc. of pituitary extract in a 4 liter beaker. This mixture was stirred slowly at room temperature for two hours to prevent the resin from settling after which it was filtered on a large Buchner funnel using No. 202 filter paper. The filtrate was designated as the A1 fraction.

The resin was washed to remove adhering follicle stimulating hormone (FSH) and inert protein by suspending it in 10 cc. of water per gm. eq. of pituitary tissue, stirring vigorously for 15 minutes and filtering. This filtrate was designated A2a and was combined with the above A1 fraction. The resin was washed again with the same volume of water and the filtrate, A2b, was discarded because it was inactive. The resin was resuspended a third and a fourth time in the same volume of water adjusted to pH 7.5 with NaOH, stirred each time for 15 minutes and filtered. These filtrates, A2c and A2d, were inactive and were discarded.

Elution of luteinizing activity from anion resin. The washed resin was suspended in

0.02*M* NaOH in the ratio of 10 cc. per gm. eq., stirred for 45 minutes and filtered. This fraction, designated A3, contained LH activity. The pH of this eluate was about 9.5. A second elution was done with the same volume of 0.02*M* NaOH, and this eluate, designated A3a, also contained LH. This fraction, which had a pH of 10.5, and the above fraction were adjusted immediately to approximately pH 7.5. These fractions were dialyzed separately against distilled water, lyophilized, and their protein content (N×6.25) determined by the method of Johnson (17).

The combined A1 and A2a fractions from the pH 8.5 adsorption contained the major part of the FSH and considerable LH activity. This fraction was treated at pH 9.0 with the same amount of resin per gm. eq. as used for the pH 8.5 adsorption. The adsorption, elution, dialysis, and recovery by lyophilization were the same as described above for pH 8.5.

Bioassay of the A1 fractions after LH had been adsorbed from the crude pituitary extracts at pH 8.5 and 9.0 showed that these fractions contained essentially all of the FSH of the extracts and significant amounts of LH activity. Attempts to improve the recovery of LH from the crude extract by increasing the resin concentration were unsuccessful as the FSH contamination in the LH fractions was markedly increased. Since FSH and LH are readily adsorbed between pH 5.5 and 6.5 the activity in the A1 fraction was recovered by adsorption on anion resin XE-59 at pH 6.0. The activity was obtained by eluting twice with 3.75% sodium acetate. These fractions provide a partially purified preparation for obtaining FSH by other procedures.

After the adsorption and elution were completed the anion resin was recycled for further use by treating it only with sodium hydroxide as described above.

Preparation of cation exchange resin XE-97. Cation exchange resin XE-97 in lots of 100 gm. was stirred to a homogeneous suspension in 4 liters of distilled water and allowed to settle. The fine particles which remained suspended were decanted and discarded. This procedure was repeated twice and the resin was then cycled through base, acid and base. The washed resin was mixed with 25 cc. of 1.25*M* NaOH per gm., stirred for one hour and allowed to settle. The alkali solution was decanted and the resin was suspended in water. This washing procedure was repeated until the water had no effect on red litmus paper. In the acid phase of the cycling, the resin was treated with 25 cc. of 1.25*M* HCl per gm. and it was washed with distilled water as described above for the NaOH treatment until the water had no effect on blue litmus paper. Finally the resin was treated again with NaOH as already described.

Purification of luteinizing fractions by use of a column of cation exchange resin XE-97. Fifty to 75 gm. of the activated resin was suspended in 1 liter of 0.5*M* pH 7.0 ammonium acetate-ammonium hydroxide buffer (38.3 gm. ammonium acetate and 0.19 ml. 28% ammonium hydroxide/liter) and stirred for two hours. After the resin had settled the buffer was decanted. This treatment was continued until the resin was equilibrated to pH 7.0 to 7.3. It was then washed several times with 0.05*M* ammonium acetate-ammonium hydroxide buffer because this was the lowest concentration used in developing the column. The resin was suspended in this buffer and added to the column (diameter 2.5 cm.) which contained a small pad of fiber glass in the bottom to retain the resin. After the desired height of the resin was obtained (25 cm.), it was resuspended in the column to obtain a more homogeneous distribution of the resin and allowed to settle. An additional 500 ml. of pH 7.0, 0.05*M* buffer was passed through the column. Fifty gm. eq. of each of the A3 and A3a LH fractions were combined, and dissolved in the 0.05*M* buffer by extracting five times with 10 cc. per extraction. The sample was added to the column and allowed to penetrate slowly into the resin. It was found that the total protein load for a 2.5×25 cm. column should not exceed 550 mg.

The columns were eluted by a stepwise increase in the concentration and the pH of the ammonium acetate-ammonium hydroxide buffer. The buffer sequence passed through the column was as follows: 0.05*M*(pH 7.0), 0.1*M*(pH 7.0), 0.2*M*(pH 7.5),

0.2*M*(pH 8.5), 0.2*M*(pH 8.75), 0.2*M*(pH 9.0), and 0.5*M*(pH 9.0). Each of the buffers except the first one also contained 0.02*M* NaCl. Approximately 50 cc. volumes of effluent were collected in about 90 minutes. The extinction of the fractions was determined at 280 mμ by use of a Model DU Beckman spectrophotometer. The distribution of the biological activity was determined by bioassay and the active fractions were combined, dialyzed and lyophilized. The protein content of these fractions was determined by the procedure mentioned above (17).

Biological assay. The LH fractions were assayed in intact 21-day-old male and female rats obtained from the Holtzman Company of Madison, Wisconsin. Rats obtained at 25 days of age were hypophysectomized at 27 days and the injections were started at 29 days. The volumes of the various fractions were adjusted with 0.9% sodium chloride solution so that each rat received the proper gm. eq. of dry pituitary tissue in 9 injections of 0.5 cc. each. The first injection was made on the afternoon of the first day and continued twice daily for four days with autopsy on the morning following the last injection. The ovaries, testes, ventral prostate and seminal vesicles were removed, dissected free of other tissues and weighed. The ovaries were examined by means of transmitted light for the presence of follicles and corpora lutea. The ovaries and testes of hypophysectomized female and male rats were fixed in Bouin's solution, sectioned and stained for histological study. The thyroids and adrenals were removed from the hypophysectomized rats and weighed.

RESULTS AND DISCUSSION

Experimental evidence has been obtained which shows that anion exchange resin XE-59 selectively adsorbs LH at pH 8.5 and 9.0 (Fig. 1). The ventral prostates from male rats which received 0.5 gm. eq. of the LH fractions were stimulated essentially to the same level obtained with fractions eluted at pH 5.8 and 6.5 where both FSH and LH were adsorbed. The FSH content of these fractions did not significantly stimulate the ovaries above the control level of 17 mg.

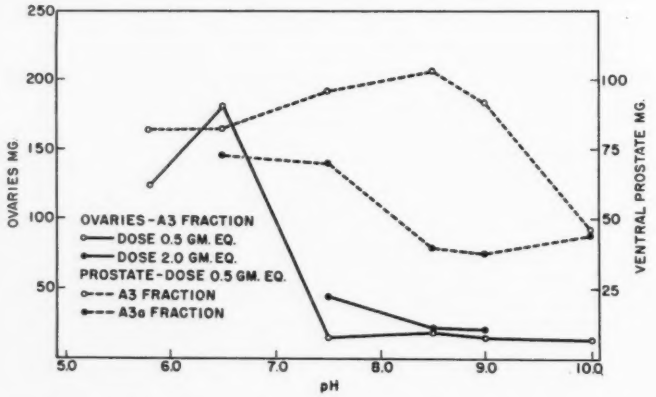

FIG. 1. Elution of pituitary gonadotrophins adsorbed on anion exchange resin XE-59 between pH 5.8 and 10.0. The resin and pituitary extract were adjusted to the pH values indicated in the figure for adsorption, and elutions were made with 0.02*M* NaOH.

The data in Table 1 are a summary of 15 LH fractions prepared by adsorption at pH 8.5, and 7 fractions prepared by adsorption at pH 9.0 from the same aqueous extract from which the pH 8.5 fractions were adsorbed. The supernatant fraction (A1) from the pH 8.5 adsorption which contained considerable LH activity was treated at pH 9.0 with XE-59 because as shown in Figure 1 LH was also selectively adsorbed at this pH. Four LH fractions were obtained by adsorbing twice with XE-59, once at pH 8.5 and once at pH 9.0, from the same aqueous pituitary extract and eluting the resin twice after each adsorption with 0.02*M* NaOH. Each LH fraction at 0.5 gm. eq., with the exception of the second elution obtained at pH 9.0, caused the prostate and seminal vesicle weights of intact immature male rats to increase a minimum of 2-fold. On the basis of the log dose-re-

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A3[1]	8.5	0.5			577 ± 9 (47)	77.1 ± 1.8	20.9 ± 0.7	5.2
A3a		0.5			585 ± 30 (25)	70.9 ± 1.9	18.1 ± 0.4	7.5
A3 + A3a		2.0	29 ± 2.3[3] (20)[4]	N[5]-FCL				
A3[2]	9.0	0.5			599 ± 10 (18)	81.8 ± 2.6	21.2 ± 0.8	5.9
A3a		0.5			575 ± 13 (16)	61.5 ± 3.4	15.4 ± 1.3	4.4
A3 + A3a		2.0	27.3 ± 2.6 (12)	N-FCL				
Uninjected controls			17 ± 0.3 (29)	N	514 ± 16 (10)	35.5 ± 0.7	8.9 ± 0.8	

[1] Average of values obtained with 15 LH preparations.
[2] Average of values obtained with 7 LH preparations.
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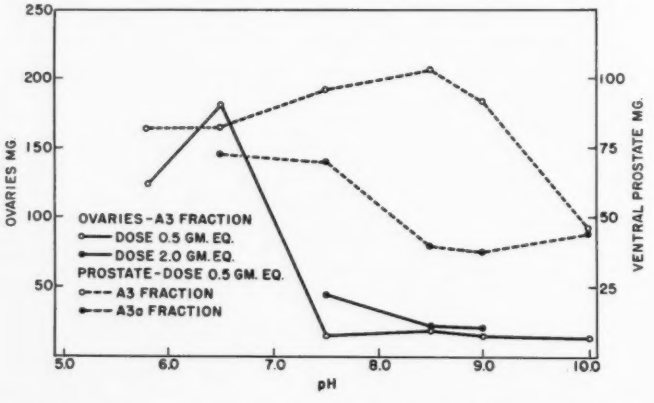

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A3+A3a		2.0	29±2.3[3] (20)[4]	N[5]-FCL				
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[1] Average of values obtained with 15 LH preparations.
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Evidence from the ovarian response of intact immature and hypophysectomized rats at 2.0 gm. eq. of the combined A3 and A3a fractions showed that the FSH concentration in these fractions is very low (Tables 1 and 3). This is further emphasized by comparing the above response with that obtained with 0.05 gm. eq. of the pH 9.0 aqueous pituitary extract used in preparing the LH fractions (Table 3). When the LH fractions were combined with FSH and administered to immature intact female rats, the ovarian response showed that these fractions were capable of augmenting the action of FSH (Table 4).

The adrenal weights obtained from the hypophysectomized female rats show that these glands undergo some hypertrophy at a dose of 2.0 gm. eq. The adrenal hypertrophy may reflect ACTH contamination. The adrenals from hypophysectomized male rats were not hypertrophied, but this can be accounted for on the basis that the males received only one fourth the dose given to the hypophysectomized females. The thyroids from the female rats indicate the presence of little, if any, TSH in these preparations.

The anion resin XE-59 is thus an effective tool for obtaining fractions which are essentially luteinizing in action. Numerous LH preparations have been prepared by this method. The procedure is simple and does not involve numerous salt and/or organic solvent fractionations required by other methods which have been reported for obtaining LH. Occasionally LH fractions obtained at pH 8.5 or 9.0 were contaminated with FSH. It

TABLE 2. ACTIVITY OF LUTEINIZING FRACTIONS AS MEASURED IN NORMAL MALE RATS

Dose[1]		Testes, mg.	Ventral Prostate, mg.	Sem. ves., mg.
Gm. eq.	Protein, mg.			
0.025	0.325	530 ± 12(17)	49.4 ± 2.0	11.3 ± 0.3
0.05	0.65	545 ± 13(17)	57.5 ± 2.5	12.9 ± 0.5
0.1	1.27	568 ± 11(17)	62.8 ± 2.1	14.3 ± 0.5
0.25	3.2	560 ± 12(17)	69.9 ± 3.0	16.9 ± 0.7
Uninjected controls		514 ± 16(10)	35.5 ± 0.7	8.9 ± 0.8

[1] Combined A3 and A3a fractions obtained with anion resin at pH 8.5. Average protein content 12.7 mg. per gm. equivalent.

was found that this FSH can be removed by dissolving the combined A3 and A3a fractions in distilled water (15 cc. per gm. eq.) and repeating the procedure with XE-59 at pH 8.5.

Further purification of the LH fractions from the anion resin was effected by adsorption on columns of cation exchange resin XE-97 equilibrated to pH 7.0 followed by elution of the hormone by a stepwise increase in buffer concentration and pH in the presence of 0.02*M* sodium chloride (see *Purification of luteinizing fractions by use of a column of cation exchanger resin XE-97* under Materials and Methods). Under these conditions, the LH activity was eluted in two peaks. The first peak (fractions 11 to 50, Fig. 2) appeared early and contained the major portion of the activity and the second peak (fractions 67 to 83) appeared near the end of the elution and contained only a small amount of activity. A small but varying amount of LH activity was present in fractions 1 to 3 which showed high absorption at 280 mμ.

Protein determinations on the fractions collected from the columns showed that approximately 50% of the total protein added to the column was recovered in the initial fractions, whereas the major peak of activity contained only 3 to 5% of the total protein. The LH preparations con-

TABLE 3. ASSAY OF LH FRACTIONS OBTAINED BY ADSORPTION ON ANION EXCHANGE RESIN XE-59 IN HYPOPHYSECTOMIZED MALE AND FEMALE RATS

Fractions	Dose, gm. eq.	Ovaries, mg.	Response	Adrenals, mg.	Thyroids, mg.	Dose, gm. eq.	Testes, mg.	Vent. prost., mg.	Sem. ves., mg.	Adrenals, mg.	Thyroids, mg.
A3+A3a	2.0[1]	16.7±2.0(6)	N-SmF[2]	16.9±1.8	5.1±0.1	0.5[1]	576±34(7)	55.9±2.9	29.4±2.8	15.3±1.8	4.3±0.09
Extract (pH 9.0)[3]	0.05[4]	129(2)	F-FCL	14	5						
Uninjected hypophysectomized controls.		8.9±0.2(4)	N	13.4±0.4	3.4±0.7		384±30(5)	13±1.0	7.7±1.0	14.3±1.6	4.5±0.7

[1] Protein content 12.7 mg./gm. eq.
[2] SmF = small follicles.
[3] The LH fractions were prepared from this extract by adsorption on anion resin XE-59.
[4] Protein content 120 mg./gm. eq.

TABLE 4. SYNERGISM OF THE LH WITH FSH ON THE OVARIES OF IMMATURE FEMALE RATS

Fractions	Dose, gm. eq.		Ovaries, mg.	Response
	LH[1]	FSH[1]		
FSH		0.5	63 ± 4.9(5)	F[2]
LH + FSH	0.5	0.5	149 ± 10.0(9)	S-NCL[2]
Uninjected controls			17 ± 0.3(29)	N

[1] LH fractions prepared with anion resin XE-59, protein 12.7 mg./gm. eq.; FSH fraction protein 0.83 mg./gm. eq.
[2] F = follicles, S-NCL = several to numerous corpora lutea.

tained on the average 0.56 mg. of protein per gm. eq. of dry pituitary tissue.

The first few fractions from the columns showed a high absorption at 280 mμ. but there was low absorption by the fractions comprising the major and minor peaks of LH activity. This difference in absorption can be accounted for in part by the high protein concentration in the initial fractions as compared to the low protein of the active fractions. However, when the initial fractions were diluted to the same protein concentration as the active fractions, they still showed a much greater absorption indicating that the active fractions were relatively low in groups absorbing at 280 mμ.

Assay of the major peak of activity in intact immature male rats with as

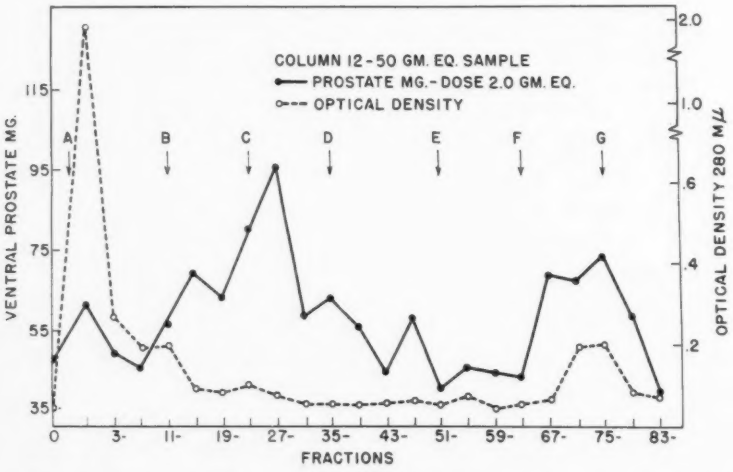

FIG. 2. Distribution of LH activity when eluted from a column of cation exchange resin XE-97. The following ammonium acetate-ammonium hydroxide buffers were added to the columns as indicated, A—pH 7.0, 0.05*M*, B—pH 7.0, 0.1*M*, C—pH 7.5, 0.2*M*, D—pH 8.5, 0.2*M*, E—pH 8.75, 0.2*M*, F—pH 9.0, 0.2*M*, and G—pH 9.0, 0.5*M*. Buffers B to G also contained 0.02*M* sodium chloride.

TABLE 5. ASSAY OF LH PURIFIED ON COLUMNS OF CATION EXCHANGE RESIN XE-97 IN NORMAL MALE RATS

LH fractions	Dose		Testes, mg.	Vent. prost., mg.	Sem. ves. mg.
	Gm. eq.	Protein, mg.			
Major peak	0.05	0.028	581 ± 26(9)	60.1 ± 4.1	13.2 ± 2.3
	0.1	0.056	624 ± 19(9)	70.1 ± 5.1	15.3 ± 0.6
	0.5	0.28	639 ± 14(9)	81.3 ± 3.9	18.4 ± 1.0
	1.0	0.56	613 ± 24(9)	87.0 ± 3.6	22.7 ± 1.6
Minor peak	0.5	0.2	621 ± 23(9)	57.7 ± 2.9	13.0 ± 1.7
	1.0	0.4	609 ± 23(9)	55.7 ± 3.9	12.9 ± 1.0
Uninjected controls			514 ± 16(10)	35.5 ± 0.7	8.9 ± 0.8

little as 0.05 gm. eq. (average of 28 μg. of protein) caused a significant increase in the weight of the ventral prostate (Table 5). When the data of Tables 2 and 5 are compared on the basis of protein content, as shown in Figure 3, the LH fractions from the cation exchange resin XE-97 are, for a given prostate weight, 37 times more active than those fractions obtained with anion resin XE-59. The combined fractions which formed the minor peak revealed a marked but low level of activity (Table 5). The ovaries were increased only slightly in weight when 2.0 gm. eq. was given.

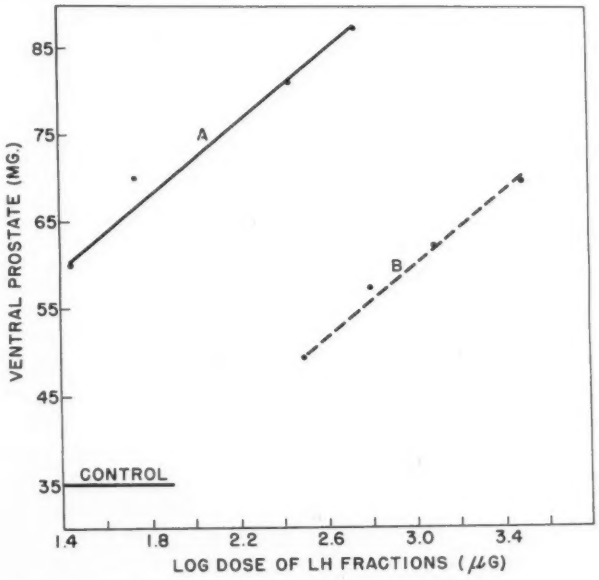

FIG. 3. Lines A and B are a comparison of the activities of LH fractions obtained, respectively, with cation and anion exchange resins. Results are taken from Tables 5 and 2. The doses are given in terms of micrograms of protein. The mean log interval is 1.57 indicating a potency of 37 to 1 for A as compared to B.

TABLE 6. ASSAY OF LH PURIFIED ON COLUMNS OF CATION EXCHANGE RESIN XE-97 IN HYPOPHYSECTOMIZED MALE AND FEMALE RATS

Fractions	Dose,[1] gm. eq.	Ovaries, mg.	Response	Adrenals, mg.	Thyroids, mg.	Testes, mg.	Vent. prost., mg.	Sem. ves., mg.	Adrenals, mg.	Thyroids, mg.
LH	0.1					468 ± 78(3)	34.8 ± 8.3	9.6 ± 1.2	13.9 ± 0.8	4.3 ± 0.8
	0.25					466 ± 23(3)	41.8 ± 1.8	12.5 ± 1.0	13.5 ± 0.1	4.9 ± 0.8
	0.5					468 ± 21(8)	36.4 ± 3.4	13.6 ± 3.9	13.3 ± 0.4	4.2 ± 0.6
	5.0	11.7 ± 0.7(8)	N	14.4 ± 1.1	4.5 ± 0.5					
FSH	0.5	71(2)	F	16	5					
FSH + LH	0.5	115 ± 2.3(3)	F-FCL	15.3 ± 1.5	4.8 ± 0.2					
Uninjected hypophysectomized controls		8.9 ± 0.2(4)	N	13.4 ± 0.4	3.4 ± 0.7	384 ± 30(5)	13 ± 1.0	7.7 ± 1.0	14.3 ± 1.6	4.5 ± 0.7

[1] Average protein content of LH fractions was 0.56 mg./gm. eq., and 0.83 mg./gm. eq. for the FSH fraction.

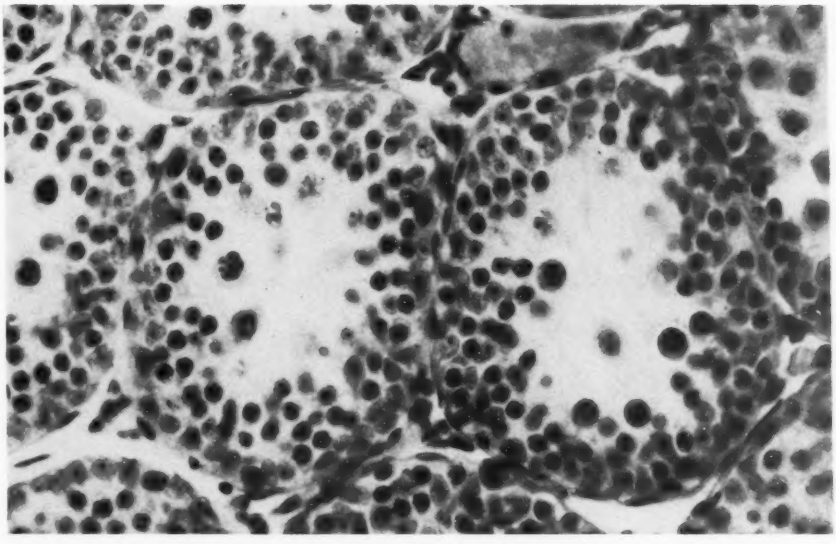

FIG. 4. Testis of hypophysectomized control male rat. (×508)

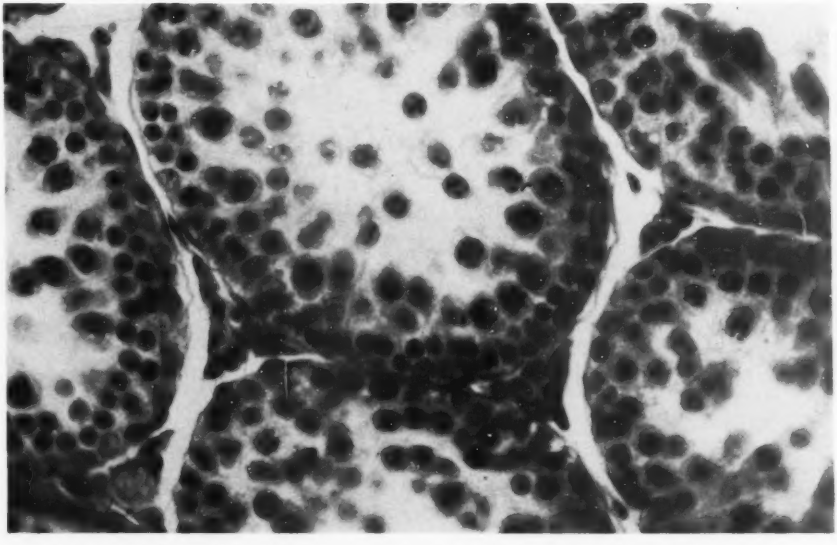

FIG. 5. Testis of hypophysectomized male rat receiving LH. Dose 0.5 gm. eq. Interstitial tissue is hypertrophied. (×508)

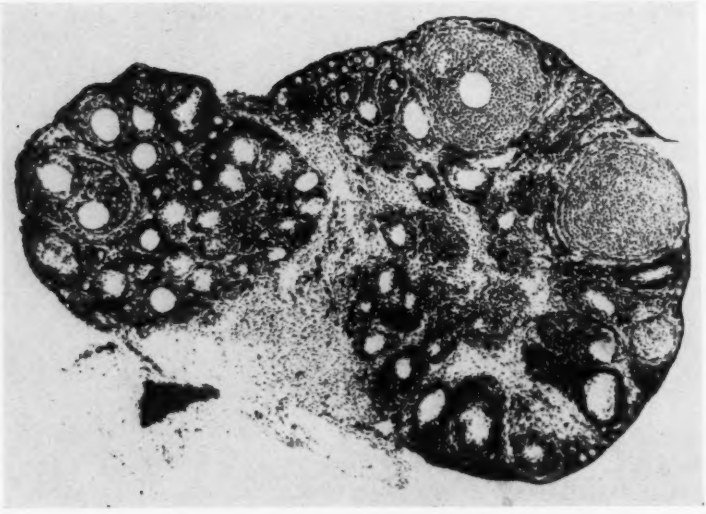

FIG. 6. Ovary of hypophysectomized control rat. (×68)

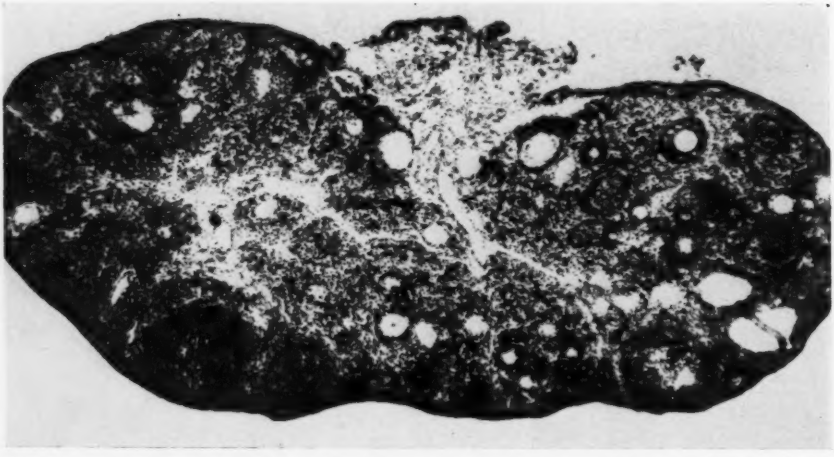

FIG. 7. Ovary of hypophysectomized rat receiving LH. Dose 5.0 gm. eq. Interstitial tissue between the follicles is increased. (×68)

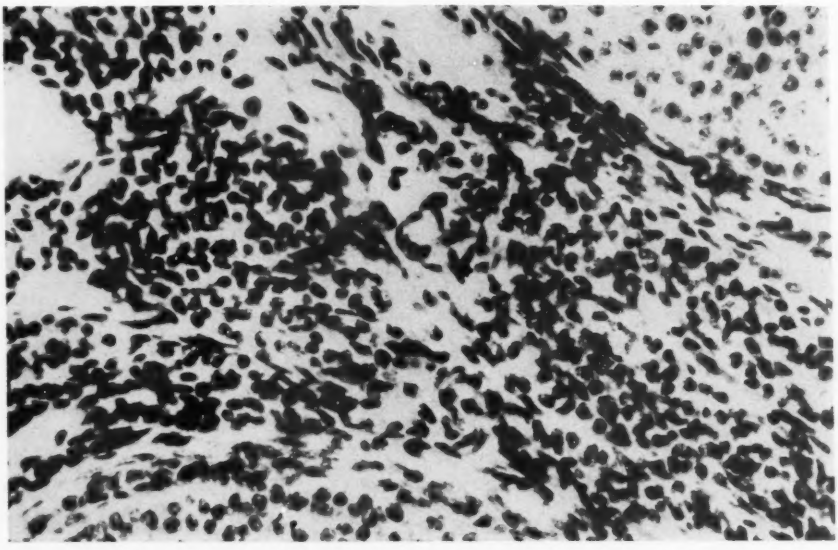

FIG. 8. Interstitial tissue in the ovary of hypophysectomized control rat. (×508)

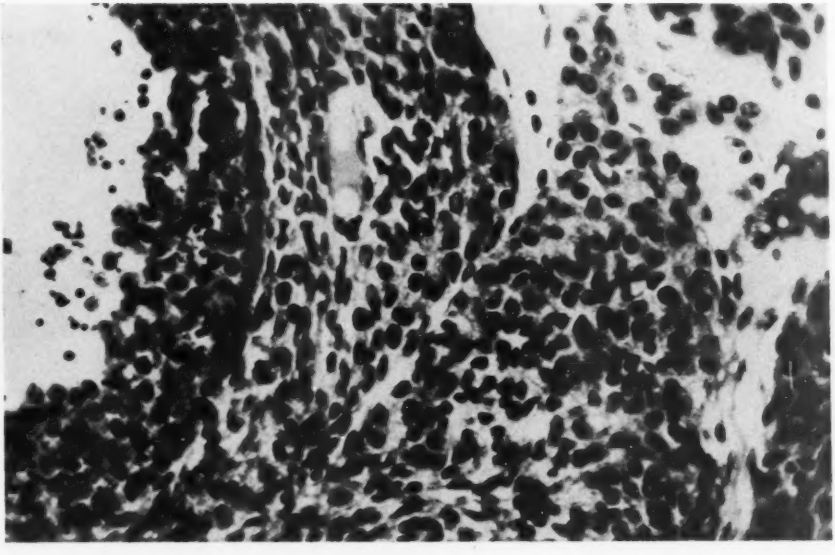

FIG. 9. Interstitial tissue in the ovary of hypophysectomized rat receiving LH. Dose 5.0 gm. Note restoration of cytoplasm and increased size of the nuclei. Follicle to the left is atretic. (×508)

FIG. 10. Ovary of hypophysectomized female rat receiving FSH. Dose 0.5 gm. eq. (×68)

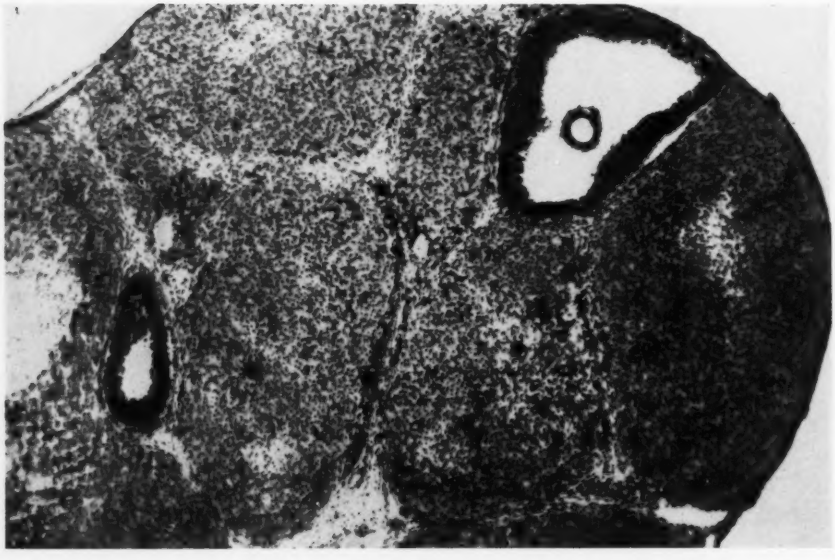

FIG. 11. Ovary of hypophysectomized female rat receiving FSH and LH. Dose 0.5 gm. eq. of each. Note corpora lutea. (×68)

The assay in hypophysectomized male rats of the purified LH fractions comprising the major peak showed that 0.25 gm. eq. (0.14 mg. protein) caused approximately a threefold increase in the weight of the ventral prostate (Table 6). The seminal vesicles also showed marked stimulation, but the increment in testes weight was small. Histological sections of the testes showed that only the interstitial tissue responded to the LH (Figs. 4 and 5). The nuclei of the interstitial tissue appeared larger and the amount of cytoplasm in the cells was increased. The seminiferous tubules were essentially unaffected.

When 5.0 gm. eq. of the LH was given to hypophysectomized female rats the ovarian response showed on the average an increase of only 3 mg. over the weight of the control ovaries (Table 6). Histological sections of these ovaries showed the characteristic repair of the deficiency condition in the interstitial cells with a restoration of cytoplasm and an increase in the size of the nuclei (Figs. 6 to 9). This probably accounts, in part, for the small increase in the weight of the ovaries. The follicles were essentially unaffected by this high dose of hormone and only a few showed the beginning of antrum formation which indicates that the LH fractions were contaminated with only very small amounts of FSH. Another possibility is that a small amount of FSH activity is intrinsically associated with LH. The LH fractions were also found to be capable of augmenting the action of FSH on the ovary and inducing the formation of corpora lutea in hypophysectomized female rats (Table 6, and Figs. 10 and 11).

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IN VITRO BIOSYNTHESIS OF ISOTOPIC ALDOSTERONE: COMPARISON OF PRECURSORS[1]

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ABSTRACT

Progesterone-4-C^{14}, desoxycorticosterone-4-C^{14}, and corticosterone-4-C^{14} are converted to aldosterone by aerobic incubation with beef adrenal capsule strippings. The yield of aldosterone from a two-hour incubation of progesterone was significantly greater than the yield from similar incubations of desoxycorticosterone or corticosterone. The specific activity of the radioactive aldosterone relative to that of the labeled precursor was also higher in progesterone incubations than in those in which desoxycorticosterone or corticosterone was used as a steroid substrate.

IT HAS been demonstrated by Giroud, Stachenko and Venning (1) and by Ayres, Gould, Simpson and Tait (2) that the synthesis of aldosterone is largely localized in the zona glomerulosa of the adrenal. In an important experiment, Ayres, Hechter, Saba, Simpson and Tait (3) demonstrated the incorporation of C^{14}-labeled desoxycorticosterone, corticosterone and progesterone into aldosterone by beef adrenal capsule strippings which contained largely zona glomerulosa tissue. In these experiments the highest yield of aldosterone was obtained when progesterone was used as the precursor. However, the relative specific activity of the isolated aldosterone was quite low (3). Incubations with corticosterone as the precursor gave the highest relative specific activity. Although interpretation of the data in terms of precursor-products relationship is necessarily limited, the results of these authors suggest that corticosterone may be an important precursor of aldosterone.

In the present experiments, C^{14}-labeled steroid precursors were incubated with beef adrenal capsule strippings prepared according to the method of Ayres and his colleagues. The results confirm the earlier reports of synthesis of aldosterone by this tissue. In agreement with the report of Ayres *et al.* (3) progesterone gave the highest yield of aldosterone. However, under the conditions of our experiments, the relative specific activity of the aldo-

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[3] This work was done during the tenure of an Established Investigatorship of the American Heart Association.

sterone isolated following incubation with C^{14}-labeled progesterone was higher than with C^{14}-labeled corticosterone or desoxycorticosterone. This finding lends support to the concept originally expressed by Rosemberg, Rosenfeld, Ungar and Dorfman (4), that progesterone may be an important precursor of aldosterone, perhaps by an alternate pathway not necessarily involving corticosterone.

METHODS

Beef adrenal glands, obtained at the slaughterhouse within three hours of the death of the animal, were kept in cold Krebs-Ringer-phosphate buffer until used. All preparative procedures were carried out at 3°. To prepare capsule strippings, the glands were bisected in their largest plane and laid capsule down on a dissection board. A knife blade was introduced into the plane of cleavage between the subcapsular tissue and the deeper zones. The capsule was flattened against the board, and a knife was moved through the plane of cleavage, cutting away the deeper zones. The thin translucent lemon-yellow subcapsular tissue was readily distinguished from the dark red deeper zones. The strippings were kept in cold Krebs-Ringer-phosphate buffer until used. About 30 gm. of tissue can be obtained from 18 beef adrenal glands in about 3 hours by this method.

About 10 grams of the capsule strippings were placed in 100 ml. of incubation medium in glass-stoppered Erlenmeyer flasks for each incubation. The incubation medium was Krebs-Ringer-phosphate buffer containing glucose (13 mM), fumaric acid (3 mM), adenosine triphosphate (1.4 mM) and nicotinamide (26 mM), finally adjusted to pH 7.4 with potassium hydroxide. Steroids were added in 0.25 ml. of ethanol per 100 ml. of medium to a concentration of 0.04 mM. The steroids used in this study were subjected to chromatography on paper to establish homogeneity.

Flasks were gassed by bubbling oxygen through the solutions for ten minutes, stoppered, and placed in a shaker bath for 2 hours at 37° C. Following the incubation the contents of the flasks were frozen for later steroid isolation.

For isolation of steroids, the contents of the flasks were thawed by shaking with $\frac{1}{3}$ volume of chloroform at room temperature, and re-extracted twice with $\frac{1}{3}$ volume of chloroform. The chloroform extracts were combined and evaporated *in vacuo* under a stream of nitrogen. The residue was partitioned between 70% ethanol and hexane. The residue from the ethanolic fraction was chromatographed on paper (propylene glycol-toluene) for 48 hours (5). The developed chromatograms were cut into $\frac{3}{4}$ inch sections and eluted with methanol for analysis in the ultraviolet.

Four fractions absorbing maximally at 240 mμ were found, regardless of the steroid incubated. The most polar of these fractions was observed inconstantly, presumably because its presence was masked by the large amounts of ultraviolet absorbing material near the origin. The second fraction in descending order of polarity had the approximate mobility of cortisol in this system, but has not been further characterized. The third fraction was slightly less polar than cortisone. The fourth and least polar fraction has not been studied.

The third fraction appeared to be aldosterone in essentially pure form. An aliquot was mixed with authentic aldosterone; the mixture chromatographed as a single band on paper in the propyllene glycol-toluene system. Rechromatography on paper in the formamide-chloroform system also gave a single band. The sulfuric acid chromogen corresponded well with that of authentic aldosterone (6). Following acetylation (acetic anhydride:pyridine 2:1, 16 hours, room temperature) the steroid migrated as a homogenous band on paper (propylene glycol-hexane). The rate of migration corresponded well

with that of authentic aldosterone diacetate. An aliquot of the free steroid was subjected to bioassay (urinary Na/K, adrenalectomized rats (7, 8)). Desoxycorticosterone acetate was used as the reference steroid. The isolated material proved to be approximately 65 times as active as desoxycorticosterone acetate, which is consistent with the known biological activity of aldosterone (9). Although there appeared to be no reason to doubt that the compound as isolated in the free form was pure aldosterone, acetylation and rechromatography were routinely used prior to quantitation as an added precaution. Quantitation was based on absorption at 240 mμ ($\epsilon = 15,\ 850$ (10, 12)). Recovery of aldosterone by this method is on the average 80%.

Radioactivity was determined with a Nuclear-Chicago D-47 gas flow counter. All counts were made in the infinitely thin range in duplicate to at least 10% reliable error.

RESULTS AND DISCUSSION

Incubations without added steroids resulted in the isolation of only trace quantities of aldosterone.

In Table 1 are presented the results of five incubations of non-isotopic steroids. Considerable variation was found between various batches of glands in the absolute amount of aldosterone synthesized. This variation may reflect differences in the condition of the glands on arrival in the laboratory or in the physiological status of the animals at time of death. However, since in each instance aliquots of the same batch of glands were distributed among the three steroid precursors, and were studied under identical conditions, comparison of the yields from the precursors is valid.

In every instance, progesterone gave a higher yield of aldosterone per millimole of steroid substrate than desoxycorticosterone or corticosterone. The differences are statistically significant (Table 1).

In Table 2 are shown the results of similar incubations of progesterone-4-C^{14}, desoxycorticosterone-4-C^{14}, and corticosterone-4-C^{14}. "Relative specific activity" indicates the ratio of the specific activity of the isolated aldosterone to the specific activity of the steroid initially added to the system. The consistently higher relative specific activity of the aldosterone derived from progesterone-4-C^{14} is noteworthy. "Per cent radioactive yield" indicates the percentage of initially added radioactivity isolated in the aldosterone fraction.

It is of interest that the yield, micromoles of aldosterone isolated per millimole of precursor incubated, was lower in these experiments than in those reported by Ayres *et al.* (3). On the other hand, the relative specific activity was higher than that reported by these workers. The discrepancies in experimental results may be related to the difference in the amount of isotopic precursor supplied to the system. Ayres and co-workers used much lower concentrations of isotopic precursors than were used in the present experiments, and their results may be explained by a proportionately greater synthesis of aldosterone from non-isotopic sources.

The greater yield of aldosterone resulting from incubation of progesterone found in our experiments is consistent with the results of Rosemberg, Rosenfeld, Ungar, and Dorfman (4), who found more chromato-

TABLE 1. ALDOSTERONE ISOLATED FROM AEROBIC BEEF ADRENAL CAPSULE STRIPPING INCUBATIONS (TWO HOURS 37°) WITH STEROID SUBSTRATES (0.04 mM)

Incubation*	Substrate	Yield	Difference	
		μM Aldosterone / mM Substrates	Progesterone minus desoxycorticosterone	Progesterone minus corticosterone
1	Progesterone	15.2	+6.5	+2.8
	Desoxycorticosterone	8.7		
	Corticosterone	12.4		
2	Progesterone	9.5	+6.3	+6.3
	Desoxycorticosterone	3.2		
	Corticosterone	3.2		
3	Progesterone	10.1	+3.0	+1.9
	Desoxycorticosterone	7.1		
	Corticosterone	8.2		
4	Progesterone	36.7	+4.2	+4.2
	Desoxycorticosterone	32.5		
	Corticosterone	32.5		
5	Progesterone	21.3	+2.9	+4.7
	Desoxycorticosterone	18.4		
	Corticosterone	16.6		
Mean			+4.6	+4.0
P Value†			<0.01	<0.01

* In each incubation the capsule strippings from a single batch of glands were simultaneously incubated with each of the indicated steroids.
† The statistical significance of these results was evaluated by analysis of paired variants.

TABLE 2. RESULTS OF AEROBIC BEEF ADRENAL CAPSULE STRIPPING INCUBATIONS (TWO HOURS 37°) WITH ISOTOPIC STEROID (0.04 mM)

Incubation*	Substrate	Yield	Relative S.A.	% Radioactive yield total cpm aldosterone ×100
		μM Aldosterone / mM Substrate	S.A. Aldosterone / S.A. Substrate	Total cpm substrate
6	Progesterone-4-C^{14}	12.4	1.03	1.28
7	Desoxycorticosterone-4-C^{14}	18.4	0.66	1.21
8	Corticosterone-4-C^{14}	10.6	0.49	0.52
9	Progesterone-4-C^{14}	7.6	0.56	0.43
	Desoxycorticosterone-4-C^{14}	3.6	0.41	0.15
	Corticosterone-4-C^{14}	12.1	0.39	0.47
10	Progesterone-4-C^{14}	34.8	0.72	2.51
	Desoxycorticosterone-4-C^{14}	18.0	0.66	1.19
	Corticosterone-4-C^{14}	12.3	0.31	0.38

* Each incubation was carried out on a different batch of adrenal capsule strippings. In incubations 9 and 10 the three steroid precursors were simultaneously incubated with the same batch of strippings.

graphically isolated aldosterone-like material in the perfusates of isolated calf adrenal after addition of progesterone to the medium than after addition of desoxycorticosterone or corticosterone. Kahnt, Neher, and Wettstein (13) have reported the conversion of desoxycorticosterone-21-C^{14} to labeled aldosterone by beef whole adrenal gland homogenates. In a single experiment Wettstein, Kahnt, and Neher (6) found no augmentation of aldosterone production by a relatively high concentration of progesterone in aerobically incubated beef whole adrenal gland homogenates.

The total amount of information available from our experiments and those reported in the literature does not justify any conclusion concerning intermediates other than the apparent fact that under certain experimental conditions progesterone, desoxycorticosterone, and corticosterone can be converted to aldosterone. Although corticosterone and desoxycorticosterone are clearly incorporated into aldosterone, the findings reported here are consistent with, though not proving, the concept that an alternate pathway of synthesis by way of progesterone and other unknown intermediates may be concerned.

Acknowledgments

We wish to express our gratitude to Drs. Ayres, Simpson and Tait for their generous advice on the preparation of beef adrenal capsule strippings. We also wish to acknowledge a gift of radioactive corticosterone from the Endocrine Study Section, National Institutes of Health, U. S. Public Health Service.

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SEX DIFFERENCE IN RATE OF RING A REDUCTION OF Δ^4-3-KETO-STEROIDS *IN VITRO* BY RAT LIVER[1,2]

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ABSTRACT

Homogenates of livers from adult female rats reduce Ring A of Δ^4-3-keto-steroids at rates 3 to 10 times greater than those from males. This large sex difference has been observed for all substrates so far tested: aldosterone, desoxycorticosterone, hydrocortisone, cortisone, corticosterone, testosterone and progesterone. It consists of a much greater concentration of the microsomal Δ^4-5α-steroid hydrogenases in the livers of females and does not involve any sex difference in availability of reduced pyridine nucleotides, nor the presence of activators in the females or inhibitors in the males.

Castration increases and testosterone decreases Δ^4-steroid hydrogenase activity in males. In females, neither castration nor estrogen administration had appreciable effect. However, a striking non-gonadal process leads to an increase in activity in both sexes during growth. The sex difference is still present, although diminished, in animals castrated when young—before the difference has appeared—and then allowed to grow to adulthood. The full adult sex difference consists predominantly of this non-gonadal induction of the enzymes, which is more marked in the female. A superimposed testosterone suppression of this process in the male increases the difference still further.

The sex difference in hepatic steroid metabolism first appears approximately at the time of appearance of the sex difference in adrenal cortical weights, but treatment of adult rats with corticosteroids does not significantly increase the Δ^4-steroid hydrogenase activity in the liver if the adrenals are present.

The greater capacity of the female rat to inactivate Δ^4-3-keto-steroids by Ring A reduction, compared to that of the male, may cause the larger size of the adrenal cortex in the female—through greater stimulation of ACTH release by more rapid removal of circulating Δ^4-3-keto-steroids from the blood. This hypothesis is strengthened by the finding of a reversed sex difference in hepatic steroid metabolism of golden hamsters, in which the males have larger adrenals than the females.

RECENTLY attempts have been made to assess the physiological importance of variations in the rate of inactivation of the adrenal cortical hormones (2, 3, 4). Only a few studies have been made so far of specific metabolic adaptations involving enzymes which catalyze the inactivation of Δ^4-3-keto-steroids. The difficulties encountered in relating changes in

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[1] A preliminary report has appeared (1).

endocrine state to changes in tissue enzyme concentrations have been clearly described (5), but the importance of the attempt to demonstrate an enzymatic basis for endocrine phenomena has been made equally clear (6, 7).

These studies were undertaken to examine physiological factors which affect the rate of hepatic inactivation of Δ^4-3-keto-steroids by Ring A reduction, and have led to the discovery of a large sex difference in the rate of the reaction *in vitro*. Because livers of female rats were found to have as much as ten-fold more Δ^4-steroid-hydrogenase activity than those of males, we attempted to relate the sex difference in adrenal cortical weight in the rat to this sex difference in enzymatic activity in the liver. We have concluded that the rate of adrenal cortical secretion may be very largely determined in many instances by the capacity of the liver to inactivate Δ^4-3-keto-steroids by reduction of Ring A, and that adaptations involving changes in activity of these enzymes frequently provoke appropriate changes in the rate of adrenal cortical secretion.

METHODS

Livers were obtained from male and female cats, rabbits, guinea pigs, golden hamsters, Wistar rats and Sprague-Dawley rats. Most of the studies were done on the two strains of albino rats, which were maintained on Purina laboratory chow and tap water *ad libitum*.

Dilute 0.25 *M* sucrose homogenates of liver (7.5 ml. sucrose/gm. of liver wet weight) were used as the source of Δ^4-steroid hydrogenases. After the homogenates were prepared, a standardized prewarming period of 5 minutes at 38° C was used, before the assay itself was begun. All homogenates were prepared and then assayed in duplicate by methods previously reported in detail (2). The preparation of the homogenates and the six-minute incubations were completed, and extractions of the remaining unmetabolized substrates begun within 25–45 minutes after the death of the animals.

In the few experiments requiring alterations of the assay, or fractionation of the homogenates by differential centrifugation, the details of the modifications are presented with the appropriate results.

Reference units for activity

Since the unit of activity employed, μM substrate reduced/gram of liver (wet weight)/15 min., is an expression of the concentration of enzyme in the liver, and not an expression of the total amount of enzyme present, in all experiments the total hepatic activity was also determined. The statistical significance of differences between experimental groups was the same for both forms of expression of hepatic activity. For convenience, only the changes in enzyme concentration are presented in detail.

Data presented as means are accompanied by the standard errors of the means. The numbers under the bars in the figures indicate the number of animals individually studied.

RESULTS

Characteristics of the assay

As we have shown previously (2), the amount of substrate reduced at Ring A during 6 minutes at 38° C in these assays is linearly related to the amount of enzyme present, and is independent of substrate concentration

and co-factor (reduced triphosphopyridine nucleotide) concentration. Duplicate assays agreed within 5% of the smaller value. Recovery of known amounts of steroid substrates from reaction mixtures in which metabolism had been prvented by chilling, or by omission of TPNH, was $95 \pm 0.7\%$ complete.

Sex difference in Ring A reduction in adult rats

The homogenates prepared from livers of female rats of both Wistar and Sprague-Dawley strains reduced Ring A of all the substrates tested three to ten times more rapidly than homogenates prepared in precisely the same manner from livers of male rats. The sex differences in Δ^4-steroid hydrogenase concentration in livers from Wistar rats is shown in Figure 1. Similar results have been obtained in 32 males and 55 females of the Sprague-Dawley strain.

The sex difference is greatest for desoxycorticosterone and corticosterone, and least for the more highly oxygenated steroids—cortisone, hydrocortisone and aldosterone. In livers of male rats the reaction rate is approximately the same (1.0 μM/gm./15 min.) for all seven substrates examined. However, in the females of both strains the reaction velocity for desoxycorticosterone is the most rapid, and is over 2.5 times greater than that of the slowest reaction (Δ^4-reduction of hydrocortisone).

The sex difference in Δ^4-3-keto-steroid reduction at Ring A is as great for those steroids secreted primarily by the gonads (testosterone, progesterone) as it is for the characteristic glucocorticoids and mineralocorticoids of the adrenal.

Cofactor requirement for Δ^4-steroid hydrogenases of males and females

The dilute homogenates used in these assays were incapable of reducing Ring A of Δ^4-3-keto-steroid substrates without the addition of reduced tri-

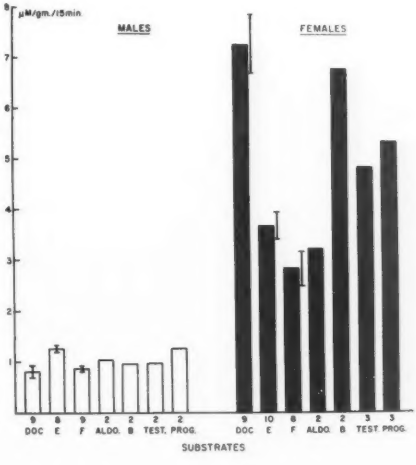

FIG. 1. Sex difference in rate of Ring A reduction of Δ^4-3-keto-steroids by Wistar rat liver homogenates. Substrates are: desoxycorticosterone (DOC), cortisone (E), hydrocortisone (F), d,1-aldosterone-21 monoacetate (Aldo), corticosterone (B), testosterone (Test), progesterone (Prog).

TABLE 1. COFACTOR REQUIREMENTS FOR Δ^4-STEROID HYDROGENASE ACTIVITY IN DILUTE CRUDE HOMOGENATES OF RAT LIVER

Sex	Substrate	Cofactor additions			
		None	DPNH 1.7×10^{-4} M	TPN 1.7×10^{-4}M +G-6-P and ZF*	TPN 1.7×10^{-4}M +G-6-P and ZF +DPNH 1.7×10^{-4}M
Female	F	0	0	2.6 (2.6†)	2.4
	E	0	0	3.4	3.0
	DOC			5.7	5.9
Male	E	0	0	0.9	0.8
	DOC	0		1.4 (1.4‡)	1.0

Activity units: μM/gm./15 min. (homogenates).
* Glucose-6-phosphate and glucose-6-phosphate dehydrogenase.
† TPNH 1.7×10^{-4}M added directly (no G-6-P or ZF).
‡ Concentration of TPN, G-6-P and ZF doubled.

phosphopyridine nucleotide (TPNH) or oxidized triphosphopyridine nucleotide and a system to reduce it enzymatically (Table 1). Reduced diphosphopyridine nucleotide was completely inactive when added alone, and it did not significantly affect reaction rates in the presence of TPNH, either by competing for the enzyme or by blocking the action of nucleotidases.

The reaction rate was not affected by doubling the amount of the TPNH-generating system added to the homogenates of male livers, and so a greater endogenous supply in the female of glucose-6-phosphate dehydrogenase, or of TPNH itself, does not seem to be involved in the sex difference in Δ^4-steroid hydrogenase activity.

Activators and inhibitors

To examine the possibility that homogenates of female livers contained an activator, or that those of male livers contained an inhibitor, homogenates of livers from each sex were assayed separately and as a 1:1 mixture, with cortisone as a substrate. The reaction velocity in the homogenate from the male was 1.8 μM/gm./15 min. and in that from the female was 2.7 μM/gm./15 min. The calculated sum of the activities was therefore 4.5 μM/gm./15 min. The measured activity of the 1:1 mixture was 4.7 μM/gm./15 min. The difference between the measured and calculated values is within the range of experimental error.

Sex difference in enzyme stability

The activities of homogenates of livers from both sexes were completely stable for at least 2 hours at 0–3° C. However, when the homogenates were prewarmed at 38° C for various periods before the assay was begun, the activity of homogenates from female livers fell rapidly to the level of ac-

tivity of homogenates from male livers (Fig. 2). In contrast, the activities of homogenates from males, for the same substrates, were completely stable during prewarming at 38° C for similar periods. Castration of adult males for several weeks tripled the amount of the activity, as described below, but also caused a qualitative change in the stability of the activities in the crude homogenate. After castration, the high levels of activity in the males showed the lability at 38° C characteristic of the activity in normal females.

The addition of TPNH and the substrate stabilized the activity at 38° C, and once the reaction had begun the velocity was constant for at least ten minutes, regardless of the extent of the loss of activity before the reaction was started. All the sex differences described are slightly underestimated by partial decay of the activity of the females during the prewarming period.

Localization of the sex difference in the microsomal Δ^4-5α-steroid hydrogenases

Ring A reduction involves two stereospecific enzymes, a microsomal Δ^4-5α-hydrogenase, and a soluble Δ^4-5β-hydrogenase (8). In the intact male rat, hydrocortisone is metabolized to both the 5α (allopregnane, A/B trans) and 5β (pregnane, A/B cis) Ring A-reduced products (9). We therefore attempted to determine whether the sex difference in metabolism involved the microsomal or the soluble enzyme. For this purpose the usual dilute sucrose homogenate, which is a 10,000×g supernatant, was divided

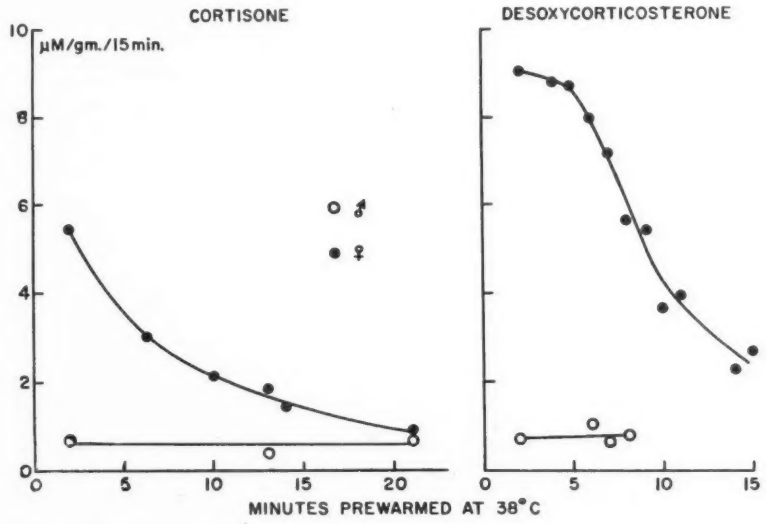

FIG. 2. Sex difference in stability of Δ^4-steroid hydrogenases during prewarming of crude homogenates at 38° C before assays started.

into three aliquots. One part was stored at 3–5° C until preparation of the other two parts was comp eted. A second part was centrifuged at 100,000×g for one hour at 5° C. The microsomal pellet obtained was resuspended in its own supernatant by hand homogenization in a McShan all-glass homogenizer. This part was used to control the effects of centrifugation and resuspension on the activities. The third part was similarly treated, except that the microsomal pellet was resuspended in a volume of cold 0.25 *M* sucrose equal to the volume of supernatant from which it had been obtained. The original supernatant was then studied separately. All four preparations were assayed simultaneously 2 hours after death of the animals. Desoxycorticosterone was used as substrate. The results are shown in Table 2.

TABLE 2. COMPARISON OF ACTIVITIES OF MICROSOMAL AND SOLUBLE Δ^4-STEROID HYDROGENASES IN MALE AND FEMALE RATS

Desoxycorticosterone metabolism (μM/gm. of liver wet wt./15 min.)

	Standard crude 0.25 *M* sucrose homogenate (10,000×g. supernatant)*	100,000×g. microsomal pellet resuspended in its own supernatant	100,000×g. microsomal pellet resuspended in 0.25 *M* sucrose (Δ^4-5α-hydrogenase)	100,000×g. supernatant (Δ^4-5β-hydrogenase)
Female	7.4	5.8	3.8	0
Male	0.7	1.2	0.5	0.7

* Sediment inactive.

When the microsomes were separated by centrifugation and then resuspended in their own supernatant, there was a significant loss of activity in the females, and a possible increase in the males. Nevertheless, the sex difference was still very evident. When the microsomes from the females were suspended in pure sucrose, instead of in sucrose containing the original soluble material of the cells, there was further loss of activity, even though the supernatant itself had no detectable activity at this high dilution. This result suggests that some soluble cofactor, in addition to TPNH, may be required by the Δ^4-5α-hydrogenases of the females.

In the male fractions, the activities of the microsomes and the supernatant assayed separately equalled in sum the activity of the fraction in which the two had been recombined. No evidence of an undefined cofactor was found.

These results indicate that in our assays the high activity of homogenates of livers from females resides entirely in the microsomal Δ^4-5α-hydrogenase, and that the soluble Δ^4-5β-hydrogenase activity is not detectable. In contrast, the low activities of homogenates of livers from males consist of low and approximately equal activities of both the microsomal and soluble enzymes. The large sex difference in the crude homogenates therefore

consists of a very much greater amount of the microsomal enzyme in the females.

These results were further confirmed by a partial examination of end-products.

Sex difference in end-products

The reaction products of the crude homogenates were chromatographed in a toluene: 70% methanol (aqueous) system for 39 hours and then stained with blue tetrazolium. Only about 50% of the quantity of hydrocortisone reduced at Ring A by homogenates from males or females could be detected by treatment with blue tetrazolium. Presumably the other half of the Ring A-reduced products were reduced at the C_{20}-carbonyl group also.

There was a definite sex difference in the Δ^4-reduced, C_{20}-unreduced products. Homogenates of livers from males reduced hydrocortisone to at least three distinct compounds more polar than both hydrocortisone and pregnane 3α,17α,21-triol-11,20-dione (THE). The major component of the three was less mobile than pregnane-3α,11β,17α,21-tetrol-20-one (THF), and possessed the approximate mobility of allopregnane-3β,11β,17α,21-tetrol-20-one in this system.

Homogenates of livers from females reduced hydrocortisone to only one Δ^4-reduced, C_{20}-unreduced component, which was not further resolved by this system. This single component possessed a mobility greater than THF but less than THE, and identical with that of one of the minor products obtained from the males. The mobility of this component was approximately that of allopregnane-3α,11β,17α,21-tetrol-20-one.

When the reaction products from livers of females and males were mixed and chromatographed, only three separate components were detected. One was the major product detected for the males, another was one of the minor products for the males, and the third apparently consisted of the single major product for the females plus the other minor product for the males.

From such preliminary studies it is possible to conclude definitely only that the major Δ^4-reduced, C_{20}-unreduced end-products are different in the two sexes. However, the interpretation given, that the major product of this type for the males is the 3β,5αisomer, while that for the females is the 3α, 5αisomer is consistent with the demonstration that livers of male rats reduce the 3-keto group of C_{19} steroids predominantly to the 3β product, *in vitro*, while livers of females reduce the same substrates predominantly to the 3α products (10).

Since there were three readily separable Δ^4-reduced, C_{20}-unreduced reaction products for the males, it seems very likely that some 5β reduction of Ring A did occur, as was also indicated by the fractionation experiments described above.

Development of the sex difference in rate of steroid Ring A reduction during normal growth

Several litters of Wistar rats 18 days old were mixed, and divided into five groups of males and females. These groups were studied at 18, 34, 40 50 and 70 days of age during normal growth in the laboratory. The results are shown in Figure 3.

At the time of weaning (18 days) there was no significant sex difference in steroid Ring A reduction between 5 males and 5 females for desoxycorticosterone, cortisone, or hydrocortisone metabolism. By the 36th day of age the vaginae of the females began to open, but the sex difference in steroid metabolism had not yet appeared. By the 40th day, when the sex difference in adrenal cortical weights first can be detected in this strain (11), the activity of livers from females for desoxycorticosterone metabolism had shown an abrupt increase, and within the period of 40–50 days of age a sex difference was apparent for metabolism of all substrates. On the 60th day of age estrus cycles began, and they were followed for two complete cycles. At the end of that time (age 70 days) the full adult sex difference in steroid metabolism was clearly established.

These changes with normal growth involved increases in the activity of livers of females, but the level of activity in male livers was not significantly different at 70 days from the value at 18 days of age.

Non-gonadal component of the sex difference

We repeated the growth curve of the hepatic Δ^4-steroid hydrogenase activity on male and female Wistar rats which had been castrated at 30

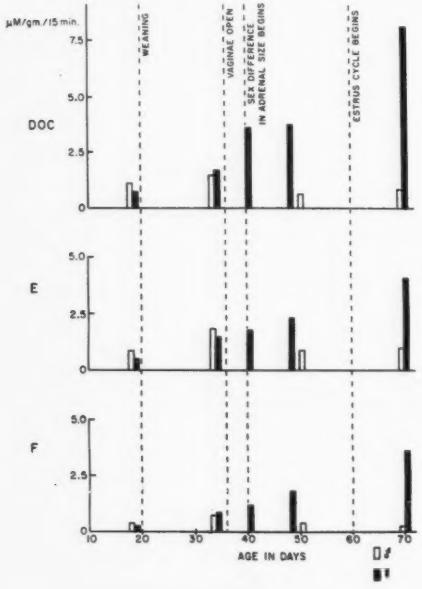

FIG. 3. Development of the sex difference in hepatic Δ^4-steroid hydrogenases during normal growth in Wistar rats. The dotted lines indicate the approximate times (within 48 hours) of some of the prominent events during maturation. Each bar represents the mean value of data obtained from 2 to 5 animals.

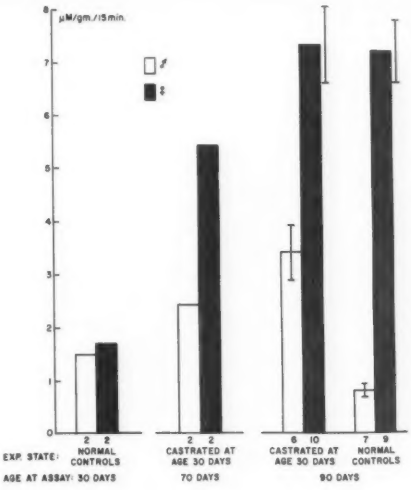

FIG. 4. Development of the sex difference in hepatic Δ^4-steroid hydrogenases in Wistar rats castrated before it had appeared, and then allowed to grow. Desoxycorticosterone was used as substrate.

days of age, well before the sex difference in steroid metabolism first appears. These castrated animals were allowed to grow, and were studied at 70 and 90 days of age. Desoxycorticosterone was used as substrate. The results are shown in Figure 4. During the growth from 30 to 90 days of age the Δ^4-steroid hydrogenase activity increased rapidly in both sexes, but the increase was more pronounced in the females, so that the sex difference was again apparent on the 70th day. By the 90th day the females had developed the same high levels of activity that would have been developed had their ovaries been present, even though the vaginae did not open, and estrus cycles were prevented.

The activity of livers from the adult males which were castrated when young reached a level four-fold greater than that of normal males of the same age, but did not reach the high levels of activity observed in adult normal or castrated females.

The castration of rats at 30 days of age, before the sex difference in adrenal weights had appeared, prevented the later development of this morphological difference. The adrenals of the males and females castrated at 30 days of age represented 0.017 ± 0.002 and $0.020 \pm 0.001\%$ of total body weights at 90 days of age. The adrenals of normal, 90 day old males and females comprised 0.012 ± 0.001 and $0.027 \pm 0.002\%$ of total body weight.

Gonadal components of the sex difference in steroid metabolism

Since the non-gonadal component of the sex difference in steroid metabolism does not account for all of the normal adult difference, we examined the effects of castration and sex hormone treatment in mature rats. The results are shown in Figure 5.

Normal adult males treated with 1 μg/day of estradiol benzoate for two

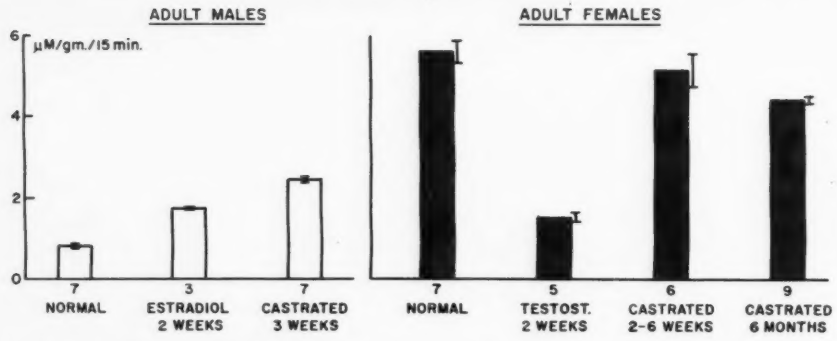

FIG. 5. Effects of castration or sex hormone treatment on the sex difference in hepatic Δ^4-steroid hydrogenases in adult rats. Sprague-Dawley rats were used. Desoxycorticosterone was the substrate.

weeks showed a significant increase in hepatic Δ^4-steroid hydrogenase activity. However, since castration of adult males for several weeks also caused a significant increase in activity, it was not certain whether the estrogenic effect was primary, or whether it was secondary to inhibition of testosterone secretion. The estrogen treatment was therefore repeated on male rats which had been castrated at 28 days of age. These animals received 1 μg./day of estradiol benzoate from the 46th to the 60th day of age. On the 60th day of age the hepatic activity was examined and found to be nearly double that of control castrated animals which did not receive estrogen treatement (Fig. 6). A true estrogenic induction of the hepatic Δ^4- steroid hydrogenase activity in males appears to be possible.

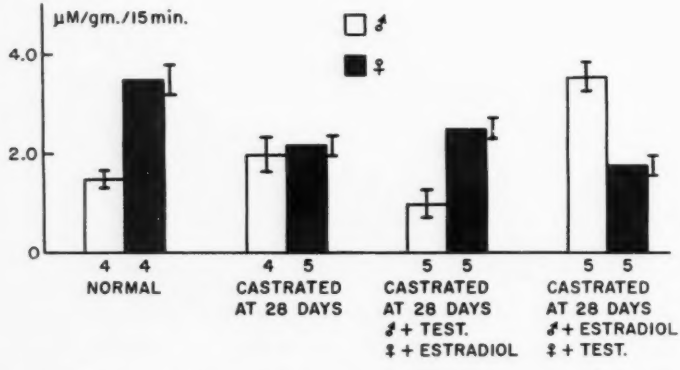

FIG. 6. Effects of castration—alone and combined with sex hormone treatment—on hepatic Δ^4-steroid hydrogenase activity in young rats. All groups were 58–62 days of age at the time of assay. Sprague-Dawley rats were used. Desoxycorticosterone was the substrate.

Testosterone treatment (1 mg/day of testosterone proprionate for 2 weeks) of intact adult males caused a diminution of activity to levels approximately 50% of normal.

Castration of adult females for two to six weeks had no significant effect on the capacity of the liver for *in vitro* reduction of steroid Ring A. Six months after castration the hepatic activity was slightly but significantly diminished (Fig. 5, $p. < 0.01$).

The effect of castration on immature females was more pronounced (Fig. 6). In the females castrated at 28 days of age and allowed to grow to 60 days of age the hepatic enzyme concentration did not increase at the usual rate with advancing age. It appears that during the growth of young females the ovaries may initiate a sudden increase in hepatic Δ^4-steroid hydrogenase activity between the 40th to 60th days of age (Fig. 3), but after that time the non-gonadal process dominates, and leads to the development of the very high adult levels of activity, whether or not the ovaries are present to cause the initial, earlier increase of the activity (Fig. 4).

Testosterone treatment (1 mg./day of testosterone proprionate for 2 weeks) caused a profound diminution of activity in normal adult females, which was clearly independent of any possible suppression of ovarian function (Fig. 5).

Before the non-gonadal process is fully developed it is possible to abolish the sex difference by castration of both sexes; to re-establish it by treatment of males with androgens, and females with estrogens; or to reverse it completely by treatment of males with estrogens and females with androgens (Fig. 6). However, after the non-gonadal process is well established, only the testosterone suppression of the enzymatic activity in males remains as a physiologically important gonadal effect on hepatic reduction of Ring A of Δ^4-3-keto-steroids.

Effects of the estrus cycle on steroid Ring A reduction

In 12 animals the rates of metabolism of desoxycorticosterone, cortisone and hydrocortisone were approximately 10% higher in diestrus than in estrus, but the differences were not statistically significant.

Role of the adrenal cortex in the non-gonodal component of the sex difference

If the larger adrenal cortex of the female rat has a greater secretory rate than does the smaller adrenal cortex of the male, then it could be supposed that at the time the morphological sex difference in the adrenals first appears, a parallel sex difference occurs in the hepatic Δ^4-steroid hydrogenases by substrate induction. We therefore attempted to increase the activity of the liver of males by injection of large doses of adrenal cortical hormones.

A group of adult male rats was adrenalectomized and treated with 5 mg./day of cortisone acetate for ten days, beginning immediately after the

operation. At the end of the treatment the hepatic concentration of Δ^4-hydrogenase activity was only about one-half that in livers of sham-operated, untreated control animals, but the livers had nearly doubled in size. Consequently, the total amounts of the hepatic enzymes for cortisone, desoxycorticosterone and hydrocortisone Ring A reduction were essentially unaffected by adrenalectomy plus immediate treatment with large doses of cortisone.

Another group of adult males was adrenalectomized and maintained on 0.3 *M* NaCl for two weeks.

Adrenalectomy and salt maintenance had no effect on the concentration of the Δ^4-steroid hydrogenase activity for cortisone, hydrocortisone, or desoxycorticosterone. The livers were 15% smaller than those of normal control animals, however, and so a slight reduction of total hepatic enzyme content may have occurred.

A similar group of male animals adrenalectomized for two weeks received a single subcutaneous 10 mg. dose of cortisone acetate, and were assayed 11 hours later. The concentrations of the Δ^4-hydrogenases in livers of this group were approximately double those in livers of normal animals, or of adrenalectomized animals which did not receive the steroid treatment. Even though these animals had received only cortisone, the increase in activity was observed for all three substrates tested. Apparently, substrate induction of these enzymes cannot be readily demonstrated, except in animals in which the substrates have been absent for some period of time. In such animals the induction may occur within a few hours.

Relationship between the sex difference in adrenal weights and the sex difference in steroid metabolism

In most groups studied the effects of the treatments on the weights of the adrenals were the same as the effects of hepatic content of Δ^4-steroid hydrogenases.

The sex difference in hepatic activity was abolished in adult rats by treating the males with estrogens and the females with androgens (Fig. 5). The adrenal sex difference was also abolished in these groups.

The sex difference in hepatic activity between young castrated groups of males receiving testosterone and females receiving estradiol was qualitatively the same as that of normal adult rats (Fig. 6). The adrenal difference was also the same as that of the adults. Furthermore, both sex differences were reversed together in such young castrated groups when the males received estrogens and the females androgens.

In one group there was a dissociation between the effects of a treatment on the weight of the adrenals, and on the hepatic capacity for Ring A reduction of Δ^4-3-keto-steroids. The animals castrated at 30 days of age and allowed to grow developed the large non-gonadal difference in hepatic activity, but did not have any sex difference in adrenal cortical weights

at 90 days of age. The opposite dissociation, consisting of a sex difference in adrenal weights in the absence of a difference in the liver enzymes, was observed in similarly prepared animals assayed one month earlier, at 60 days of age.

Hepatic Δ^4-steroid hydrogenase activity in other species

We also prepared homogenates from livers of adult male and female pairs of cats, golden hamsters, guinea pigs and rabbits. The results obtained from these homogenates, with cortisone as substrate, are shown in Figure 7, and are compared to activities of livers from one pair of Wistar and Sprague-

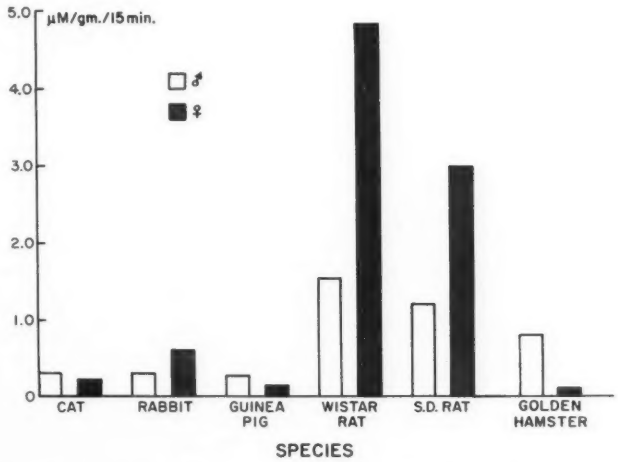

FIG. 7. Hepatic Δ^4-steroid hydrogenase activity in male and female pairs of several species. The sex difference in steroid metabolism in golden hamsters is the reverse of that in the rats. Cortisone was used as substrate.

Dawley rats assayed at the same time. Homogenates from the other species had much less activity than those from rats, and it was not possible to evaluate confidently the difference between the males and females by means of the dilute homogenates and short incubation times employed.

Because hamsters have a reversed sex difference in adrenal weights, compared to that of rats (12), we tested further the significance of the reversed sex difference in steroid metabolism shown in Figure 7. When the assay was repeated with desoxycorticosterone as substrate, the same reversed sex difference shown in Figure 7 for cortisone was observed: the activity of the livers of males was 0.75 μM/gm./15 min., while that of livers from females was only 0.20 μM/gm./15 min. We then increased the reliability of the assay for low levels of activity by doubling the concentration of the

homogenates, by extending the incubation period from the usual six minutes to ten minutes, and by making all measurements in triplicate. The activity in four males, with desoxycorticosterone as substrate, was 0.89 μM/gm/15 min., while that of four females was only 0.19 μM/gm./15 min. ($P < 0.01$). Adrenals of the males comprised 0.019% of total body weight, while those of females comprised only 0.014% ($P < 0.01$).

DISCUSSION

Any physiological interpretation of the impressive *in vitro* sex difference in steroid metabolism revealed by these experiments has to be advanced with caution until it is proved that this difference affects the biological half-life of Δ^4-3-keto-steroids *in vivo*. Our demonstration of the same sex difference in steroid metabolism by surviving slices of rat liver (13) is a strong reason to believe that this sex difference in Ring A reduction shown by liver homogenates does represent a difference inherent in the intact livers.

Since our results appear to exclude activators, inhibitors, and cofactors as the basis for this difference in hepatic Δ^4-steroid hydrogenase activity, we have concluded that it originates in the enzymes themselves. The simplest view consistent with all our data is that livers of females contain 3 to 10 fold more of the microsomal Δ^4-5α-steroid hydrogenases than those of males, and that the activity of the soluble Δ^4-5β-steroid hydrogenases is low in dilute homogenates of livers from males, and not detectable in those from females. Both the results of fractionation of the homogenates by differential centrifugation (Table 2) and the preliminary examination of end-products support this interpretation of the sex difference.

The remarkable lability at 38° C of the Δ^4-steroid hydrogenase activity from livers of females, and the stabilization of the activity after addition of substrate and TPNH, suggest that release of some proteolytic activity by homogenization may occur. Since 38° C is the normal temperature of rat liver, it is unlikely that a thermolability of the enzymes could account for the loss of activity. There is a remote possibility that livers of males may have contained as much activity before homogenization as did those of females and that through more rapid proteolysis after homogenization an artificial *in vitro* sex difference was created. This possibility is apparently excluded by the finding of the same sex difference in steroid metabolism by surviving liver slices (13).

Other quantitative sex differences in hepatic metabolism in rats have been described (14–16), but none is as large as this difference in rate of reduction of Ring A of Δ^4-3-keto-steroids.

Attempts have been made to explain the sex difference in adrenal size in the rat (17–22). Most previous studies have been concerned with the effects of sex hormones on adrenal size, on rate of synthesis of corticosteroids, on sensitivity to ACTH, or on ACTH release itself. No clear explanation is

yet available, and most hypotheses previously advanced, which attribute the sexual dimorphism of the adrenal to the actions of the sex hormones themselves, are embarrassed by the fact that the adrenal dimorphism is reversed in the golden hamster.

Our finding of a large sex difference in the capacity of the livers of female and male rats to inactivate Δ^4-3-keto-steroids *in vitro* by reduction of Ring A suggests a new explanation of sex differences in adrenal size: the rates of ACTH release and adrenal cortical secretion may be determined by the rate at which Ring A of circulating corticosteroids is reduced in the liver. If such is the case, the feed-back stabilization of circulating levels of corticosteroids should not be attributed to the "hypothalamic-pituitary-adrenal" axis, but rather to the hepatic-hypothalamic-pituitary-adrenal axis.

This hypothesis led us to predict that the hamster, with a reversed sexual dimorphism of the adrenal gland compared to that of rats, should have to show a reversed sex difference in hepatic steroid metabolism also. This prediction was confirmed by our results. Furthermore, we have previously demonstrated that there is an extraordinarily close correlation ($r = 0.97$) between the size of the adrenal gland and the capacity of the liver to inactivate cortisone by reduction of Ring A in normal, hypothyroid, and hyperthyroid rats (13). It follows then, that the relationship between the liver and the adrenal may be of general importance—even in other species which do not happen to manifest the relationship overtly by a sex difference in adrenal size and hepatic enzyme content.

If, as we suppose, the sex differences in steroid metabolism found in rats and hamsters actually do cause the sexual dimorphism of the adrenals, it is

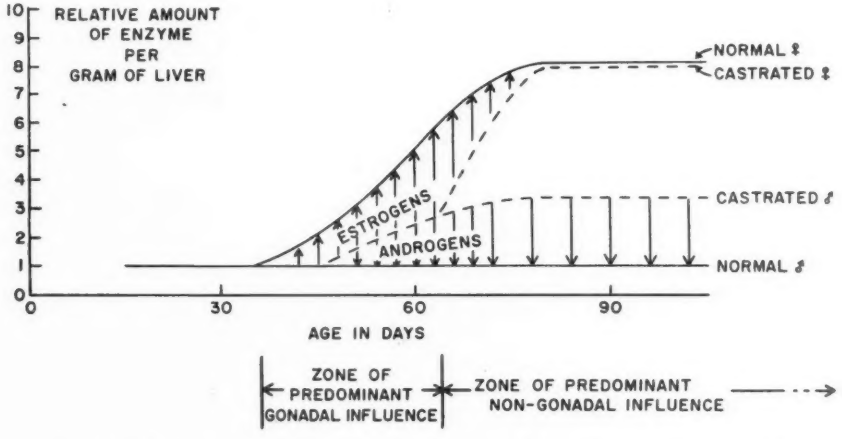

FIG. 8. Schematic summary of the factors involved in the development of the sex difference in hepatic Δ^4-steroid hydrogenases in rats.

then necessary to account for the sex difference in the hepatic enzymes themselves. A morphological difference in one organ has merely been translated by our hypothesis into an enzymatic difference in another.

Figure 8 shows a schematic summary of our present interpretation of the data concerning the development of the sex difference in steroid Ring A metabolism in the rat. The difference is initiated by an estrogenic induction of the enzymes in livers of maturing females. This stage of estrogenic induction is followed shortly by a non-gonadal induction of the activity in both sexes, which in the maturing males is completely suppressed by the gonadal androgens. Finally, the estrogen sensitivity is later diminished or lost in the females, and the non-gonadal process becomes sufficient by itself to maintain the high levels of activity. In contrast, the androgen inhibition in the males persists unabated after it first appears.

In the adult rat the major part of the sex difference is not dependent upon the presence of functioning gonads, but stems from some non-gonadal process. Our data seem to exclude substrate induction of Δ^4-steroid hydrogenases by adrenal cortical secretions as the basis for the large non-gonadal component of the sex difference, and its nature remains unknown.

Acknowledgments

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THE EFFECT OF PARATHYROIDECTOMY ON HAVERSIAN REMODELING OF BONE[1]

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ABSTRACT

The occurrence of haversian remodeling has been measured in control and parathyroidectomized adult dogs given a tracer dose of calcium-45. Autoradiographs, microradiographs and histologic sections were made of samples of diaphyseal and metaphyseal bone and the numbers of resorption cavities and newly formed haversian systems compared for the control and experimental animals. The results indicate that haversian remodeling is reduced in the parathyroidectomized animals. Measurements of the actively calcifying lengths of the systems show that the reduction in the remodeling incidence in the experimental animals is not the result of a shorter length undergoing calcification but of a smaller number of systems being formed. Two of the dogs used in the experiment were females that had born and weaned a litter six weeks previously. In both these animals there is increased bone remodeling compared with the male dogs, however, the parathyroidectomized female shows a reduced incidence of remodeling compared with her female control.

THAT there is an interrelationship between the parathyroids, osteoclastic activity, and resorption of bone is well established, even though the details of the relationship are not all known. The parathyroid glands monitor the calcium ion concentration in the blood plasma, and respond to a diminution in this concentration by increased activity, leading in turn to increased resorption of bone with consequent release into the blood of dissolved calcium. These are component parts of a feedback mechanism, concerned in the homeostatic regulation of the calcium ion concentration in the fluids of the body.

The resorption of bone and the subsequent rebuilding, referred to as remodeling, is of two sorts: 1) the remodeling incident to growth; and 2) the internal reconstruction of compact bone, known as haversian remodeling. The remodeling of growth is especially prominent at the junctions of the epiphyses of the long bones with their shafts where, in the metaphyseal regions, a reshaping of the bones continues throughout their growth in

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length. While it has been shown that parathyroidectomy leads to a partial failure of the normal resorption pattern in this region, there is not a complete cessation of osteoclastic activity.

The present paper is concerned with the internal, or haversian remodeling of compact bone, which occurs in two main stages: 1) a tunneling process, occurring apparently at random in compact bone, leading to the formation of resorption cavities; and 2) the filling in of these tunnels or cavities by the laying down of concentric layers or lamellae of bone, resulting in the formation of new haversian systems. It is this remodeling in adult compact bone that plays an important part in the maintenance of a constant calcium ion concentration in the blood, and thus performs an important physiological function. It occurs throughout the life of the animal and in man even appears to increase in old age.

Little is known about the control of haversian remodeling. The tunneling operation resembles resorption in other areas of bone, and is accompanied by the presence of osteoclasts; when construction of a new haversian system begins these are replaced by osteoblasts, and new bone formation proceeds in the usual fashion. It has seemed desirable to determine whether the resorption incident to the formation of cavities in compact bone, to be later filled in by new haversian systems, is wholly or partly under the control of parathyroid activity. To this end littermate adult dogs have been thyro-parathyroidectomized, and the effects upon the internal structure and remodeling of cortical bone have been observed and compared with control animals.

METHODS

A litter of four 2-year old dogs was used, three of which were males, and one of which was a female that had borne a litter of 4 puppies weaned six weeks before the experiment began. As a control for this female a mongrel of the same age that had weaned 4 puppies six weeks previously was used. Two males and the one female of the litter were thyro-parathyroidectomized, four parathyroids being identified from each animal. The serum calcium and phosphorus of the experimental animals were determined before and after operation; there was a sharp drop in the calcium level and a rise in serum phosphorus after removal of the parathyroids. Both the control and experimental animals were given 5% calcium chloride intraperitoneally and fed on a high calcium diet for 12 days after the operation. The animals were kept for two months without further treatment to permit the bone tissue to be stabilized.

Two female dogs, one experimental and one control, were given a single injection of Ca^{45} and killed a month after injection; one male experimental dog was injected once again a month later and killed a week after the second injection; two male dogs, one experimental and one control were injected three times with Ca^{45} every two months and killed one week after the last injection. It was hoped that a comparison between animals given one injection and those given multiple injections would give an indication of the rate of bone building. However, in this respect, no great differences were observable between the animals that had received single and multiple injections.

As the dogs were killed the femurs and tibia-fibulas were removed and fixed in 100% alcohol. A piece approximately one and one half inches long was cut from each tibia

shaft, the mid point being the point of union of the tibia with the fibula; this piece served as the sample of tibial diaphysis (Table 1). Each femur shaft was transected at the mid point, and a half inch distal to this was preserved as the sample of femur diaphysis. Beyond this a quarter inch length was decalcified and prepared for histologic sections. Distal to this another half inch block served as the sample of femur metaphysis. The samples of tibial diaphysis, femur diaphysis and femur metaphysis were embedded in methyl methacrylate and cut into cross sections 100μ thick and 500μ apart.

Microradiographs and low resolution autoradiographs (Kodak Spectroscopic Plate Autoradiographic Type A) were made of the sections. From the autoradiographs the number of actively calcifying osteons in each section was counted. An actively calcifying system was taken to be demonstrated by an increase in darkening of the film by a factor of three or more times that of the diffuse reaction. An estimation of the area of each section was made and the number of calcifying osteons per square centimeter for each of the three pieces of bone from each dog obtained.

TABLE 1. NUMBER OF CALCIFYING OSTEONS/SQ. CM. OF SECTION

	Femur diaphysis	Femur metaphysis	Tibia diaphysis	Average length of calcifying osteon
♂ Experimental	11	18	14	0.66 mm.
♂ Experimental	11	22	26	0.71 mm.
♂ Control	31	52	72	0.74 mm.
♀ Experimental	45	67	160	0.92 mm.
♀ Control	78	213	366	0.88 mm.

The number of calcifying osteons/sq. cm. appearing in each section could vary because the actual number of different osteons varied or because the length of the osteons varied. To find out if the variations in the number of osteons were the result of changes in length or of the appearance of new systems, the length of the calcifying osteons was measured in the diaphyseal blocks of the femurs and tibias of the dogs. Calcifying osteons of lengths less than 500μ were taken to be 500μ long; this would result in a slightly higher value for the average length if many osteons are much shorter than 500μ, but since the results are comparable rather than absolute, no error is incurred. The values for the average number and length of the actively calcifying region of osteons in the diaphysis of the femurs and tibias, and the metaphysis of the femurs of the five dogs comprise the results of the experiment. (Table 1).

The disappearance of Ca^{45} from the blood of the five dogs was measured after each injection and curves drawn which proved to be identical for the experimental and control animals.

RESULTS AND DISCUSSION

A comparison of the values given for the three pieces of bone demonstrates the differences in turnover in the bones and in different regions of the same bone. The tibia diaphysis shows a consistently higher remodeling rate than the femur diaphysis, and the metaphysis of the femur has a higher rate than the diaphysis by a factor of two or more. However these variations between the different bones and regions of the same bone are consistent in the five dogs and the significant variations appear between the control and experimental animals.

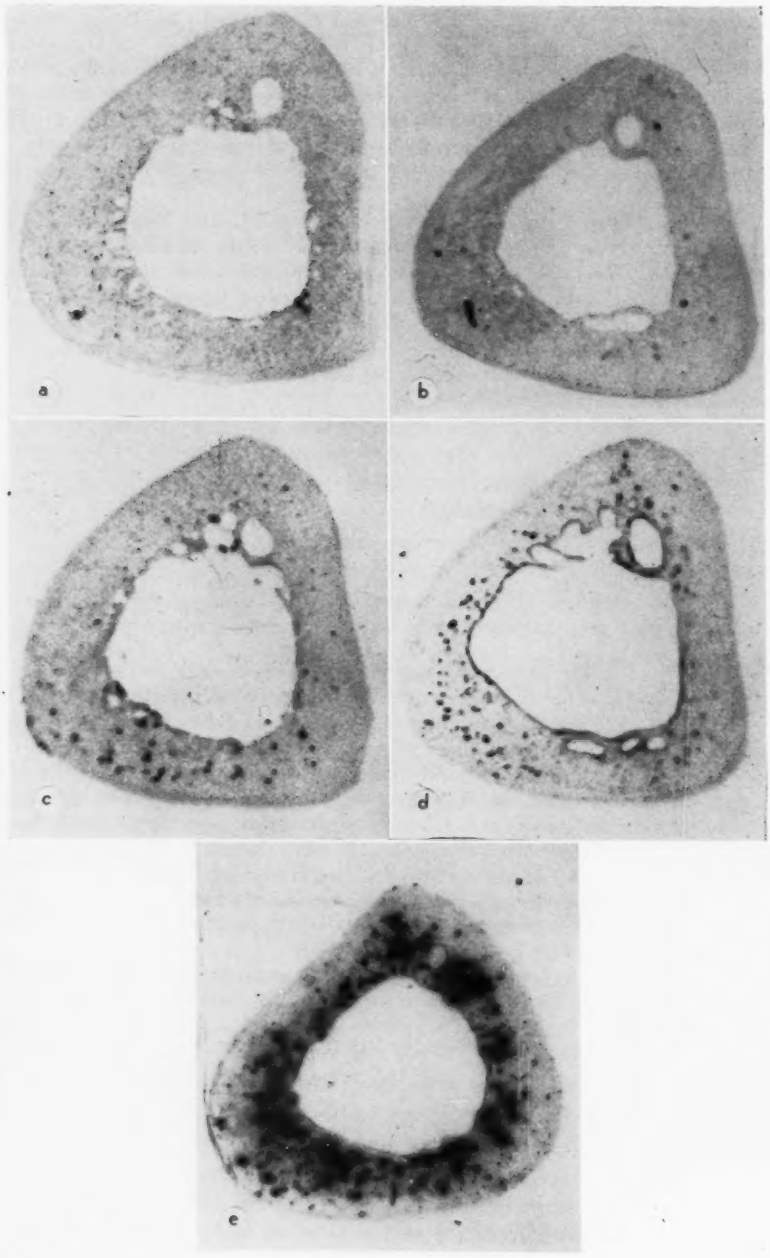

FIG. 1. Autoradiographs of mid-diaphyseal tibial sections from the experimental and control dogs. Magnification×7. a) Experimental male. b) Experimental male. c) Control male. d) Experimental female. e) Control female.

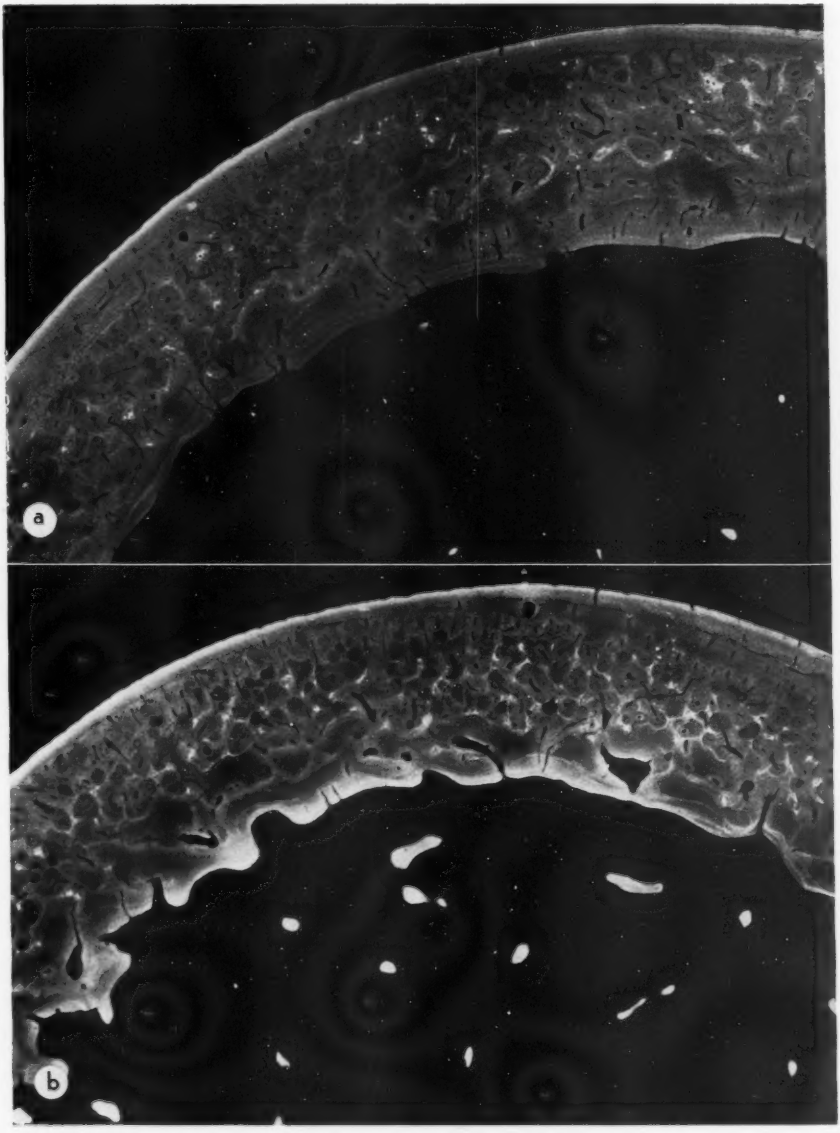

FIG. 2. Microradiographs of mid-diaphyseal femoral sections from an experimental and control animal. Magnification×23. a) Experimental male. b) Control male.

The results show that the two female dogs, two months after bearing and weaning four puppies, are rebuilding their bone at a tremendous rate, however the rate of rebuilding is slowed down to a half or less of its normal value in the parathyroidectomized female compared with her control. The male dogs show the same result, namely a rate of bone formation that is a factor of two or three less in a parathyroidectomized animal when compared with the control (Table 1 and Fig. 1).

The changes in the lengths of the actively calcifying systems are not so dramatic. As values in themselves they mean little but they are comparable within the experiment and they show that the large increases in numbers of calcifying osteons in sections from the male dogs cannot be explained merely by an increase in length. Both the control and experimental female dogs show an increase in the length of the calcifying systems which must contribute somewhat to the increase in number of calcifying osteons seen in each section.

The smaller number of calcifying systems resulting from parathyroidectomy indicates that the removal of the parathyroids reduced the rate of resorption. The alternate interpretation that there was not a reduction in resorption but rather a failure of calcification would lead one to expect a larger number of resorption cavities in the experimental animals, and this was not observed. Both the microradiographs (Fig. 2) and the histologic sections show that there are approximately the same number of resorption cavities, perhaps fewer, in the sections from the experimental animals as compared with their controls. Resorption is in any case not completely prevented, and it is obvious from the results that factors other than the parathyroids influence resorption in adult cortical bone. In the two female animals there is the influence of pregnancy and lactation which apparently causes increased resorption which, though reduced in the parathyroidectomized animal, is still three or four times the incidence found in the male experimental animals. This increased resorption is reflected in the consequent increased rebuilding that occurs a month after weaning. It is perhaps worth while to point out here that the time at which there will be maximum uptake of an isotope in an adult animal is not during pregnancy but a week or more after the litter has been weaned, when repair of the bone is taking place.

Acknowledgments

We much appreciate the help of Dr. Smith Freeman of Northwestern University who thyro-parathyroidectomized the animals, and Dr. Robert Flynn who cared for the animals during the experiment. We thank Charles Williams, now at the University of Illinois College of Medicine, for his interest and conscientious technical assistance.

QUANTITATIVE HISTOCHEMICAL DISTRIBUTION OF COENZYME A IN THE RAT ADRENAL IN VARIOUS FUNCTIONAL STATES[1,2]

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ABSTRACT

Modifications of the procedure of Malmstrom and Glick for the micro measurement of coenzyme A were employed in a study of the quantitative histological distribution of the coenzyme in adrenal glands of male albino rats. Subcutaneous injection of 25 mg. ACTH per kg. 3 hours before sacrifice produced significant increases in coenzyme A concentration in the adjoining zones between the glomerulosa and fasciculata, and the fasciculata and reticularis. Small increases occurred in the medulla and the reticular-medullary border zone. Subcutaneous injection of 5 mg. calcium-d-pantothenate 3 hours before sacrifice increased the coenzyme concentration in all zones except the adjacent fascicular-reticular regions.

Analysis of whole gland homogenates prepared from adrenals of animals killed at noon and at midnight revealed that the coenzyme concentration was about 100 per cent greater in the latter. Injection of ACTH abolished day-night differences by producing maximal concentrations in both groups. Injection of pantothenate also elevated the concentration, but simultaneous administration of both ACTH and pantothenate resulted in values lower than those obtained with ACTH alone. A series of subcutaneous cortisone injections (7 mg. cortisone acetate/kg. daily for 2 weeks) produced near maximal values in both groups when the data were expressed as coenzyme A per unit protein-nitrogen. Six days after hypophysectomy the coenzyme A concentration had fallen drastically and day-night differences were minimized.

THE essential role of dietary pantothenate and of its physiologically active form, coenzyme A (CoA), for the integrity and function of the adrenal gland has been treated in various reviews, *e.g.* (1–4). Malmstrom and Glick (5) developed a procedure for the determination of millimicrogram quantities of CoA and they applied it to a study of the distribution of CoA in the normal rat adrenal. In the present study the work was extended to include some methodological modifications, and effects of ad-

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[3] Data in this paper were included in a thesis submitted in partial fulfillment of the requirements of the Graduate School of the University of Minnesota for the degree of Doctor of Philosophy.

ministration of pantothenate and ACTH on the quantitative histological distribution of CoA in the rat adrenal. In addition, extracts of whole gland homogenates were used to observe effects on CoA concentrations of hypophysectomy and injections of ACTH and of cortisone in rats which were sacrificed at noon and at midnight, periods of low and high bodily activity. Extracts of glands of rats killed at noon after treatments with both ACTH and pantothenate, as well as with pantothenate alone, were also analyzed.

EXPERIMENTAL

Adrenal glands were obtained from male albino rats (Sprague-Dawley) weighing 300–400 gm., approximately 3 months old, purchased from Holtzman Co., Madison, Wis. Sham operated and hypophysectomized rats were obtained from the Hormone Assay Laboratories, Inc., Chicago. The animals were fed Purina Fox Chow and tap water *ad libitum*. Hypophysectomized and sham operated rats were given water, milk, orange, lettuce, and canned dog food. The rats were housed in a constant climate room with controlled illumination, and sacrificed as described previously (6) at either noon or midnight ± 15 minutes.

ACTH (25 mg./Kg.) and calcium-d-pantothenate (5 mg./rat) were administered subcutaneously to separate animals in 1 ml. of physiological saline solution 3 hours prior to sacrifice. Cortisone acetate (7 mg./Kg.) was administered subcutaneously to each rat in about 0.1 ml. of aqueous suspension once daily for 2 weeks prior to sacrifice. Both hypophysectomized and sham operated rats were sacrificed 6 days after surgery.

After sacrifice the adrenals were quickly removed, frozen with solid carbon dioxide, and transferred to previously chilled plastic vials containing strips of filter paper saturated with physiological saline solution. The vials were capped and placed in a cold room at −15° C for 15 minutes. Fresh frozen microtome sections (2 mm. diameter, 16 μ thick, 0.05 μl. vol.) were then prepared as described previously (7). In serial tissue section work, one section was brushed flat on an albuminized glass slide for histological examination after toluidene blue staining (8), the next 8 sections were placed in a reaction tube containing frozen buffer solution for CoA analysis, and the following section was placed in another tube for protein-nitrogen (P_N) analysis (9). This sequence was followed throughout.

Measurement of Coenzyme A

Modifications of the procedure by Malmstrom and Glick (5) for CoA analysis included substitution of 0.5 *M* Tris buffer, pH 7.57, for 0.1 *M* phosphate, pH 7.0, and 1 *M* bicarbonate. This type of substitution was first reported by Novelli (10), and the volumes and pH were adjusted in the present procedure to give a pH of 7.0 in the final reaction mixture. Pigeon liver extract was prepared and aged according to Kaplan and Lipmann (11) from an acetone powder of pigeon liver purchased from Pentex Inc., Kankakee, Ill. Both pigeon liver extract and sulfanilamide reaction mixture were stored in 0.5 ml. aliquots in small glass vials with screw top Bakelite caps. The vials were kept at −20° C, thawed immediately before use, and used only once.

The micro chemical apparatus was essentially the same as that employed earlier (5) but the measurements were made in a Beckman DU spectrophotometer with Lowry-Bessey cuvettes for volumes > 50 μl. For small volumes (to 5 μl.) inserts for these cuvettes were used with required accessories (12). Micro homogenizers were made of glass tubes, 40 mm. long and 5 mm. internal diameter, with bottom inner surfaces, and ends of glass rod pestles, ground with carborundum paste (13, Fig. 49).

Extracts were obtained by homogenizing single fresh adrenals for 1 minute in 200 μl. of 0.5 *M* Tris buffer, pH 7.57, at 5° C, followed by placing the tubes in boiling water for 30 seconds and then in an ice bath for 1 minute, centrifuging at 10,000 *g*. for 30 seconds, and transferring 6.5 μl. aliquots of the supernatant to small reaction tubes, 2.0–2.5 mm. internal diameter, 28 mm. long. For the tissue sections a seal of 6.5 μl. of the Tris buffer was placed across the lumen of each small reaction tube a few mm. from the bottom, and frozen by placing the tubes in the cryostat microtome cabinet used for sectioning. Eight sections were transferred to the surface of the frozen buffer in each tube with the tip of a stainless steel wire. When sectioning was complete the tubes were placed in boiling water for 15 seconds and then in an ice bath for 1 minute. The tissue was pushed down into the buffer with the stainless steel wire and disintegrated and mixed by "buzzing" for 1 minute.

The common procedure for both the extracts of homogenates and section samples follows:

(1) To each tube add 4.6 μl. of 0.1 *M* cysteine hydrochloride.
(2) Add 13 μl. of a mixture of equal volumes of sulfanilamide reaction mixture and pigeon liver extract.
(3) Cap tubes and mix by "buzzing."
(4) Place tubes in a water bath at 37° C for 2 hours.
(5) Halt the reaction by transferring 6.5 μl. aliquots of digestion mixture to larger tubes (4 mm. inner diameter, 25 mm. long) containing 50 μl. of 5% trichloroacetic acid, and mix.
(6) Centrifuge for 5 minutes at 2500 *g*.
(7) Transfer 23.5 μl. aliquots of clear liquid to tubes, 4 mm. diameter.
(8) Add 2.5 μl. of 0.1% sodium nitrite, and mix.
(9) After 3 minutes add 2.5 μl. of 0.5% ammonium sulfamate, and mix.
(10) After 2 minutes add 2.5 μl. of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, and mix.
(11) Add 30 μl. of 5% trichloroacetic acid, and mix.
(12) Transfer to micro cuvettes and determine absorbance at 546 mμ.
(13) Determine absorbance of non-tissue or non-extract blanks.
(14) Calculate milliunits of CoA present from formula of Malmstrom and Glick (1954): $c = n/1-n$, where c = concentration of CoA, n = S/Sm, S = percent of added sulfanilamide acetylated, Sm = maximum of S as c approaches ∞. For any 2 pairs of c and S, calculate Sm from: $Sm = (c_2-c_1)/(c_2/S_2)-(c_1/S_1)$.

A major difficulty in developing the micro procedure was the increased rate of autooxidation of sulfhydryl groups when the volume of the digestion mixture was reduced without a proportionate decrease in the air-liquid interface. A linear relationship was found between per cent of CoA measured and cross-sectional area of the tube down to 1 $mm.^2$ which gave 100%. From this a correction may be made for the size of the tube used.

RESULTS AND DISCUSSION

Histological Distribution and Effect of Treatments

In confirmation of earlier work (5), adrenals from untreated animals gave highest values of CoA per section in the outer fasciculata and the fascicular-reticular zone, and intermediary values in the reticular-medullary zone (Fig. 1). When the data were expresssed as CoA per unit P_N the same general relations held. Administration of ACTH produced significant increases in CoA concentration in the glomerular-fascicular and fascicular-

reticular zones, and less pronounced increases in the reticular-medullary zone and medulla. Loading with pantothenate elevated CoA concentrations in all regions except the fascicular-reticular zone, however the increase exceeded that given by ACTH only in the inner fasciculata and central reticularis.

The need to use eight tissue sections to obtain sufficient material for CoA analysis obviated finer histological localization. Consequently, a more sensitive microbiological assay method is being developed in this laboratory to permit further refinement of these studies.

The data obtained are of particular interest with respect to the quantitative distribution of cholesterol compounds in the adrenal which occur in highest concentration in the outer fasciculata (14). The increase in adrenal CoA following ACTH treatment is accompanied by a decrease in cholesterol. Since CoA has been found to be involved in cholesterol synthesis,

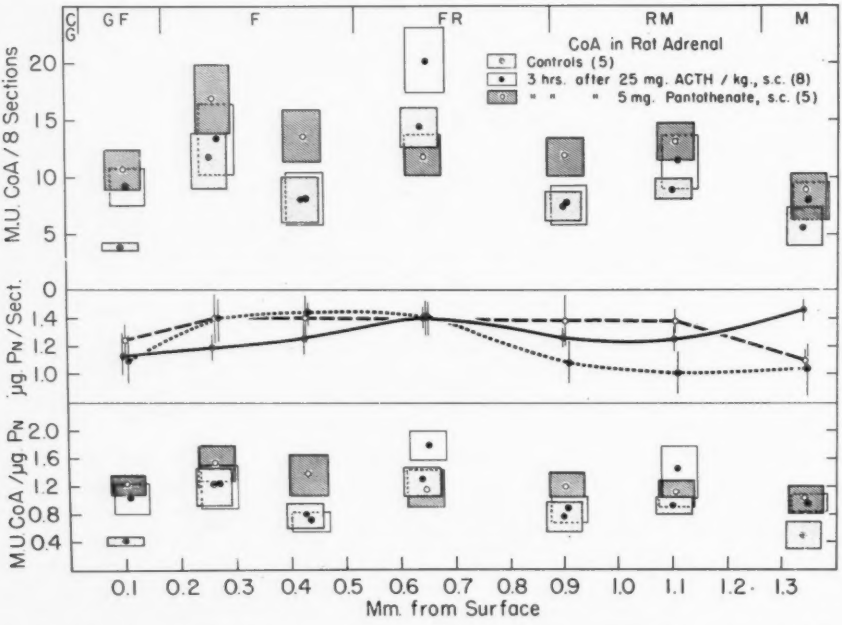

FIG. 1. Quantitative histological distribution of CoA (expressed in milliunits) in adrenal glands of male albino rats sacrificed at noon. Eight microtome sections, 2 mm. diameter, 16 μ thick, total volume 0.4 μl., used for each CoA analysis; one section used for P_N. The height of each rectangle represents twice the standard error of the mean value, the width is equivalent to the thickness of 8 sections (128 μ). The numbers in parenthesis after the treatments indicated are the number of adrenals used to obtain the composite data. Regions marked C,G,F,R and M denote, respectively, capsule, glomerulosa, fasciculata, reticularis and medulla; mixed zones are designated by both letters.

e.g. (2, 15, 16), depletion of adrenal cholesterol may elicit a compensatory response of renewed synthesis with an increase in those agents, such as CoA, required for this process. This would be compatible with the fact that ACTH stimulates conversion of acetate to cholesterol in the perfused adrenal, *e.g.* (17, 18).

CoA is also involved in other metabolic processes, and the finding of Nichols and Little (19) that slices of dog adrenal from the separate zones exhibit an increase in oxygen consumption *in vitro* on addition of ACTH may also be related to an increase in CoA. However, the succinic dehydrogenase system in the rat adrenal responded little if at all when the animals were injected with ACTH (6).

Day-Night Variations and Effect of Treatments

Comparisons of CoA data for whole rat adrenals from animals sacrificed at noon and at midnight, with and without treatments, may be made from Table 1. The time of killing had no discernible influence on the wet weight of the glands. The amount and concentration of CoA in the adrenal was significantly greater (about 100%) when the untreated animals were sacrificed at midnight, the more active period, than at noon, the relatively quiescent period. Stimulation of the adrenal by ACTH during the day evoked much greater increase in amount and concentration of CoA than during the night when the adrenal is already stimulated to some degree by the greater activity of the animal. However, the ACTH treatment resulted in maximal concentrations of CoA which were the same in both the day and the night groups.

When the adrenal was loaded with CoA by pantothenate injection, the CoA per gland was not quite as great as it was following ACTH because the gland itself was not quite as large in the former case. The concentration of CoA on the P_N basis was practically the same in both cases, but on the wet weight basis the value found following ACTH injection was greater, as would be expected from the lipide depletion. When both the ACTH and the pantothenate were administered simultaneously to the same rats, the resulting CoA values were significantly lower than those obtained with ACTH alone. The reason for this is not apparent.

The series of cortisone injections caused a drastic loss in adrenal weight as expected, and this was accompanied by a marked decrease in CoA per gland in the group killed at midnight. Only a small effect, and in the reverse direction, was observed with the group sacrificed at noon. The concentration of CoA was decidedly increased over the controls in the noon group, while no effect was demonstrable in the midnight group. However, the actual CoA per P_N in both groups was essentially the same and near maximal. It is difficult to relate these findings to those of Sourkes and Heneage (20) who injected 3 mg. of cortisone per day for 6 days into rats and found that homogenates of their adrenals exhibited reduced oxidative

TABLE 1. EFFECT OF TREATMENTS AND TIME OF SACRIFICE ON MEAN CONCENTRATIONS OF COENZYME A IN WHOLE LEFT ADRENAL GLANDS OF MALE ALBINO RATS

Treatment	No. adrenals	Mg. wet wt. / gland	± S.E.	p*	MU CoA / gland	± S.E.	p*	MU CoA / mg. wet wt.	± S.E.	p*	MU CoA / μg. P_N	± S.E.	p*
Sacrificed at Noon													
Untreated	14	27	1.2		384	33		15	1.6		10	1.0	
ACTH†	9	29	1.2	0.3	1033	74	<0.001	36	2.7	<0.001	22	2.8	<0.001
Ca-d-Pantothenate§	4	25	0.7	0.208	698	108	0.049	28	3.9	0.01	19	1.5	<0.001
ACTH and Ca-d-Pantothenate†§	9	27	1.2	>0.6	636	36	<0.001	25	1.3	<0.001	15	1.3	0.01
Cortisone‡	4	12	0.5	<0.001	503	76	0.234	41	4.8	0.002	18	2.2	0.01
Sham Hypophysectomy	4	24	1.6		1083	78		46	4.5		41	2.2	
Hypophysectomy	4	12	0.5	0.002	532	64	0.005	39	3.3	0.018	24	1.4	<0.001
Sacrificed at Midnight													
Untreated	13	26	0.7		796	68		31	2.2		18	1.6	
ACTH†	13	27	1.0	0.158	994	59	0.046	37	2.7	0.113	23	1.5	0.03
Cortisone‡	4	13	0.7	<0.001	389	25	0.005	31	1.5	>0.6	16	0.7	>0.6
Sham Hypophysectomy	4	22	2.3		1201	180		55	3.0		38	2.5	
Hypophysectomy	4	13	0.8	0.019	362	54	0.011	29	6.7	<0.001	18	3.0	<0.001

* p values referred to corresponding data on untreated rats; hypophysectomy data referred to that on sham-operated rats, animals sacrificed 6 days after operation.
† Sacrificed 3 hrs. after subcutaneous injection of 25 mg. ACTH/Kg.
‡ Sacrificed after 2 weeks of daily subcutaneous injections of 7 mg. cortisone acetate/Kg.
§ Sacrificed 3 hrs. after subcutaneous injection of 5 mg. calcium-d-pantothenate/rat.

metabolism when the data were referred to wet weight and to activity per gland. Glick and Greenberg (6) observed small increases in the activity of the succinic dehydrogenase system following a series of cortisone treatments parallel to those employed in the present study.

The high values found after sham hypophysectomy are probably due to surgical stress. It should be pointed out that these animals belonged to a different group than that employed for the other experiments, and it is likely that there were strain differences. Therefore the sham-operated and hypophysectomized rats should be compared only with each other. This comparison reveals that, with decreases in gland weight to about one-half, profound decreases in CoA occurred in all cases after hypophysectomy. Day-night differences were largely eliminated by this operation even though percentile decreases in the night group were somewhat greater than those in the day group. Hypophysectomy also decreases the activity of the succinic dehydrogenase system in the rat adrenal (6).

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FUNCTIONAL RESTITUTION OF PITUITARY GRAFTS RE-TRANSPLANTED FROM KIDNEY TO MEDIAN EMINENCE[1]

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ABSTRACT

The present study is based on the fact that rat pituitaries autografted on the kidney lose power to maintain ovarian follicles and interstitium, although luteotropic function is retained or enhanced. In 62 adult, cycling rats the pars distalis was autotransplanted to the kidney immediately after parapharyngeal hypophysectomy In the definitive experimental group of 14 rats (MEm-1) the grafts were re-transplanted after 3 to 4 weeks via the transtemporal route into close relation to the median eminence. Estrous cycles returned spontaneously in 13 animals 8 to 68 days later; 7 rats became pregnant. Ovaries, uteri and vaginae were histologically normal save for reduced numbers of follicles and corpora lutea. In another group of 10 rats (MEm-2) grafts were re-transplanted under the median eminence 2 to 3 weeks after the first operation. These were each "primed" later with a short sequence of FSH and LH injections, a treatment of little benefit. Gonadotropin secretion became evident at 11 to 66 days; 3 rats cycled while 4 became persistent-estrous; none was fertile.

In 21 control animals (group TL) in which the graft was re-transplanted from kidney to a site under the temporal lobe and in 17 rats (group Ky) in which the grafts remained on the kidney, there was no sign of return of FSH and/or LH secretion during experimental periods of 30 to 105 days.

Adrenals and thyroids were studied histologically, ACTH was evaluated functionally by adrenal hypertrophy after unilateral adrenalectomy, and TSH was evaluated by thyroid uptake of I^{131}. While residual stimulation of adrenals and thyroids was evident in control groups TL and Ky, there was significant improvement in group MEm-1. Group MEm-2 was not studied.

In the MEm group all grafts were closely adherent to the median eminence. Results clearly demonstrate that the extreme functional deficiencies observed when the pars distalis is removed to sites distant from the hypothalamus are caused by the loss of hypothalmic influences, stimuli that are probably mediated by the hypophysial portal vessels. This is true with respect to secretion of ACTH, TSH, FSH and LH.

TRANSPLANTATION of the pars distalis of the adenohypophysis to sites remote from the hypothalamus in hypophysectomized female rats results in atrophy of the ovarian follicular apparatus and interstitial

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tissue, as well as in marked reduction in thyroid and adrenocortical stimulation (1, 2, 3). Somatotropin secretion is probably retained in diminished amount. Luetotropin secretion, on the other hand, is spared and may possibly be increased (3, 4, 5), with the result that recently formed corpora lutea existing at the time of hypophysial transplantation will be stimulated to full activity. This will be indefinitely maintained.

Accompanying the apparent losses of FSH and LH activities and the deficiencies in TSH and ACTH output, there are pronounced changes in cytology of the pars distalis graft. The predominant cell types that can be recognized in routinely stained material (3, 4, 6) are judged to be small chromophobes and modified acidophiles. Recent investigation with the aldehyde-fuchsin (AF), periodic acid-Schiff (PAS) and other techniques indicate that occasional minute beta and delta basophiles are retained for long periods (7), and that the acidophiles are characteristically orangeophilic in Azan preparations (6).

Not only does one find these qualitative changes in cytology in the transplanted gland, but there is also a predictable quantitative loss of parenchyma caused by the massive central infarction (7). The healthy parenchyma of the graft derives from a relatively thin shell of tissue not involved in the infarct.

The several functional deficiencies mentioned could be ascribed to the extensive quantitative loss, perhaps in combination with unrecognized damage in the remaining parenchyma consequent to manipulation of the gland and to its temporary deprivation of blood supply. The cytologic changes could be thought of as an expression of such damaging action. Other facts, however, do not agree with that view and indicate that a close neurovascular relationship of the pars distalis to the hypothalamus is required for normal gland activities. The present study emphatically supports that contention by demonstrating the return of reasonably normal functions and cytology after the re-transplantation of a long-established renal-autograft of pars distalis to a site immediately under the median eminence.

These experiments are based on those of Greep (8), and Harris and Jacobsohn (2). Greep found reproductive functions well preserved in hypophysectomized rats if the hypophyses were simply replaced in their original sites. The impossibility of histologic control, however, lent unavoidable uncertainty to the interpretation of the result. Harris and Jacobsohn eliminated this fault by introducing the transplant directly beneath the median eminence via the trans-temporal route, immediately after parapharyngeal hypophysectomy. The hypophysial capsule thus could be searched later on for remnants. These authors used post-partum mothers as recipients in most cases, transplanting 5 to 11 pituitaries from members of the animal's own litter into each host. While such grafts placed under the temporal lobe of the brain failed to sustain ovarian function, those under the median

eminence usually became functional in a short time. Not only did estrous cycles return, but many rats experienced normal pregnancy with spontaneous parturition. With respect to thyroid and adrenal function, the somewhat limited data reported indicate "normal" maintenance by grafts under the median eminence, but complete or partial atrophy in the temporal lobe controls. Here was substantial demonstration that some influence mediated by the median eminence, presumably chemical and transmitted by hypophysial portal vessels, is essential for most normal functions of the pars distalis.

Our findings agree with this and go further by showing that an animal's own hypophysis, after losing its ability to support ovarian functions other than progestational when the gland is removed from the influence of the tuber cinereum, will regain them when this influence is restored. Analogous restoration of thyroid and adrenal function is also documented. Preliminary experiments of this kind were reported last year (9, 10) in a group of 13 rats among which 5 resumed estrous cycles and 3 became pregnant. That work has now been amplified, and most of the grafts of the experimental and control series have been studied cytologically. The cytologic features are described elsewhere (7).

Experimental plan. The general plan remains unchanged, one group of animals receiving re-transplants under the median eminence, another receiving re-transplants under the temporal lobe, and a third group retaining the grafts in the renal capsule. There were a few minor modifications. Estrogen priming was omitted, but in part of the median eminence group a brief priming with extrinsic FSH and LH was administered—unnecessarily, it seems. The definitive median eminence group was not primed in any way. In all but one animal of that group, the graft was left on the kidney for a full month before re-transplantation. Finally, any possible complications that might have been introduced by persisting functional corpora lutea were avoided by carrying out the initial transplantations to the renal capsule during proestrus (5).

MATERIALS AND METHODS

The experimental animals were 72 adult female rats from our inbred Osborne-Mendel strain, ranging in age from 3½ to 7 months at the beginning of the experiment. The animals at this time weighed between 156 and 252 grams. They were kept in a room in which the temperature ordinarily ranged from 76° to 80° F the year around. The lights were controlled by a time switch, "on" for 14 hours and "off" for the next 10 hours. The basic diet of Purina dog chow and water *ad libitum* was supplemented after the various operations by milk three times a week, brewer's yeast once a week and 5% dextrose added to the drinking water at all times.

Vaginal smears were taken at least 2 weeks preoperatively and throughout the postoperative period every day except Sundays.

The animals were distributed into 4 groups, in all but one of which the pars distalis was autotransplanted into the renal capsule. The technique of hypophysectomy and transplantation was that described by Everett (3). The operation was carried out on

the day of proestrus as a standard procedure; the only exceptions were 11 rats brought into the temporal lobe control group from the preliminary study.

Group designations and characterizations are as follows: *Group MEm* consists of 24 animals subdivided into subgroups MEm-1 (14 rats) and MEm-2 (10 rats). In *Group MEm-1*, the definitive experimental series, the grafted pars distalis was allowed to stay on the kidney 29 to 33 days (with 1 exception of 21 days) and was then re-transplanted under the median eminence. *Group MEm-2* differs only in that the grafts remained on the kidneys for a shorter time (15 to 19 days) and that these animals were briefly injected with FSH and LH beginning 7 to 28 days after re-transplantation of the graft. *Group TL* comprises 21 control animals in which the pituitary grafts were left on the kidney for 12 to 26 days and were then re-transplanted under the temporal lobe of the brain. Eight of the 21 rats were injected with FSH and LH for reasons to be given later on. *Group Ky* consists of 17 control animals in which the pars distalis was simply transplanted to the kidney capsule and left there for the duration of the experiment (31 to 93 days). *Group N* comprises 10 normal cycling animals.

The technique of the trans-temporal approach in re-transplantation of the grafts was that described by Harris (11). Special care was taken to remove adherent portions of the kidney capsule from the grafts at the time of re-transplantation. Furthermore, they were manipulated as little as possible during the transfer. For a very brief interval between removal from the kidney and insertion under the brain they were placed in 0.9% NaCl. A subcutaneous injection of 1.0 mg. cortisone acetate was administered to each animal about 10 minutes before the operation.

Secretion of ACTH and TSH was investigated in 11 animals from group MEm-1, 9 animals from group TL, 5 from group Ky and all 10 animals from group N. ACTH secretion was evaluated by the adrenal hypertrophy test. In each instance, on a given day, the right adrenal was removed and weighed, and autopsy was performed 10 days later. At that time the left adrenal was weighed, and its hypertrophy was determined by the per cent weight difference of the two glands. TSH secretion was measured indirectly by 24-hour radioactive iodine uptake in the thyroids. An injection of 1.5 μc. of I^{131} was given intraperitoneally 24 hours prior to sacrifice. At autopsy, the thyroid was removed, trimmed and weighed. Half was fixed for histologic examination, the other half was weighed and used for the iodine uptake study. From its radioactivity the uptake of I^{131} was calculated per milligram of gland and subsequently expressed as uptake per whole gland.

In the particular cases where gonadotropic hormones were administered, FSH was injected subcutaneously twice daily[3] over a period of 3 days. This was followed on the 4th day by an injection of LH.[3]

At the beginning and during the length of the experimental period, all animals were weighed once a week. At the end, the animals were killed with an overdose of Nembutal. Ovaries, adrenals and thyroids were trimmed, weighed, and fixed in Bouin's solution. A small piece of each uterus and vagina was also fixed in this medium. The base of the skull containing the hypophysial capsule was fixed in Heidenhain's SUSA overnight and decalcified by immersion in 5% trichloroacetic acid for 48 to 72 hours. All pituitary grafts were examined *in situ;* in cases of re-transplantation under the median eminence their position was recorded by a sketch of the region. All grafts were fixed in Elftman's chrome alum fixative (12) for the cytologic study (7). In the median eminence groups a piece of adjacent hypothalamus was left in contact with the graft. All tissues intended for histologic examination were imbedded in paraffin, serially sectioned and, except for pituitary grafts, stained with a modified Mallory trichrome technique.

[3] We are indebted for various FSH and LH preparations to Dr. W. H. McShan of the Department of Zoology, University of Wisconsin; to Dr. J. A. Green, Department of Anatomy, University of Indiana; and to Dr. Irby Bunding of the Armour Laboratories.

EXPERIMENTS AND RESULTS

In group MEm-1, after the grafts were placed under the median eminence, nearly every animal (13 of the 14 rats) began to cycle spontaneously. The time at which the first proestrus occurred varied widely from 8 to 68 days after operation, averaging 35 days. In a few animals the cycles were regular from the beginning, in some cases the early ones were irregular and the later ones regular, while in 3 rats they were usually irregular, with prolonged diestrous intervals. In the 10 rats having more or less regular cycles the characteristic length was 5 days. There appeared to be no correlation between cycle regularity and the rapidity of post-operative return of estrus. There was some correlation with fertility, however.

All 13 cycling animals were placed with known fertile males at the approach of estrus on one or several occasions. Seven accepted coitus and became pregnant, whereas 6 failed on repeated exposures. Among the latter were the 3 rats with irregular and prolonged cycles. The fact of pregnancy in the 7 rats is of interest chiefly in that it establishes that these cycles were truly productive of normal eggs and new corpora lutea and that hormone balance during the progravid phase was adequate for nidation. Although autotransplantation of pars distalis to the kidney on the day after ovulation and coitus will often allow pregnancy to continue, nidation near the normal time requires administration of a small amount of estrogen (13, 14).

Three of the pregnant rats delivered spontaneously at the end of 23 days. One had not given birth on the 24th day and was delivered artificially. Possibly the remaining 3 animals would have delivered spontaneously had the fetuses not been removed by caesarian section shortly before term. The size of the litters was smaller than normal in every case, ranging from 1 to 6 pups (1 had 6 pups, 4 had 5, 1 had 2, and 1 had a single one). None of the mothers was able to nurse her young, although the mammary glands appeared to be well developed.

In group MEm-2, primed with FSH and LH, the intent of this hormonal treatment was to build up the atrophied ovaries so that they might possibly respond more rapidly to gonadotropin secreted by the graft. This hypothesis proved wrong, however, and final results were somewhat less satisfactory than in group MEm-1. In 7 of the 10 rats, gonadotropin secretion became apparent 11 to 66 days after re-transplantation of the graft, averaging 36 days. The other 3 rats remained anestrous. In 4 of the 7 rats, persistent estrus ensued immediately or at a short interval after the FSH-LH treatment, while 3 began to cycle. Only one rat had more or less regular cycles, however, the others being distinctly irregular with variable prolongation of diestrus. Mating trials were carried out in only 2 of these cycling animals, including the one with "regular" cycles. She became pregnant after the 4th trial, but later resorbed the litter. The other rat failed to become pregnant, although sperm were found in the vagina after the second trial.

All animals from control groups TL and Ky remained anestrous throughout the experimental period. Animals from the normal control group N experienced regular estrous cycles throughout the time of observation.

Ovaries. In Table 1 the mean ovarian weights of the various groups of animals can be compared. It is seen that although the animals in group MEm-2 were treated with gonadotropins the weight of their ovaries was no

TABLE 1. EFFECT OF LOCATION OF THE PARS DISTALIS ON OVARIAN WEIGHT

Group	No. of rats*	Ovarian wt. mg./100 g. (mean ± S.E.)	P
TL	20	4.3 ± 0.31	<0.01
Ky	5	6.1 ± 0.42	<0.01
MEm-1	11	17.9 ± 1.3	
MEm-2	6	15.5 ± 8.15	<0.01
N	10	26.6 ± 1.3	

* Since most of the animals in group Ky were eventually used in other experiments, their ovarian weights could not fairly be entered here. In other groups as well, certain individual cases were not appropriate and are excluded from the table.

greater than those in group MEm-1. The large standard error in group MEm-2 is caused by the fact that the weight of ovaries of animals with persistent estrus was very low owing to the lack of corpora lutea. It is obvious from the table that the mean ovarian weights of animals from groups MEm-1 and MEm-2 were lower than in the normal animals, but considerably greater than those of control groups TL and Ky.

Histologic comparison of ovaries from the various groups was even more instructive than the comparison of their weights (Figs. 1, 2, 3 and 4). In groups TL and Ky, histologic examination showed complete atrophy of both the follicular apparatus and the interstitial tissue (Fig. 5). The few corpora lutea that were ever found were small and seemingly inactive ones, containing relatively large numbers of stromal cells. The aspect of ovaries from group MEm-1 varied with the time of the cycle at which the animal was killed, but in general showed excellent restoration. Growing follicles were always found, and the interstitial tissue was well stimulated in many places (Fig. 6), although there were always some zones that remained atrophic. The aspect of ovaries from the 3 animals of group MEm-2 that cycled, even irregularly, was similar to that of animals from group MEm-1. However, ovaries from the animals with persistent estrus were small and composed mainly of large follicles, being devoid of corpora lutea. Interstitial tissue was generally similar to that of the cycling animals.

Correlated with the fact that weights of ovaries in groups MEm-1 and

MEm-2 were somewhat less than in normal rats, the numbers of follicles and corpora lutea in given sets were usually less than normal. This was expressed in the pregnant rats by the small numbers of fetuses.

Evidence of a significant degree of gonadotropin secretion was seen in the ovaries of the four MEm rats that failed to cycle. The interstitial tissue in local areas was clearly stimulated. Correlated with this was a moderate

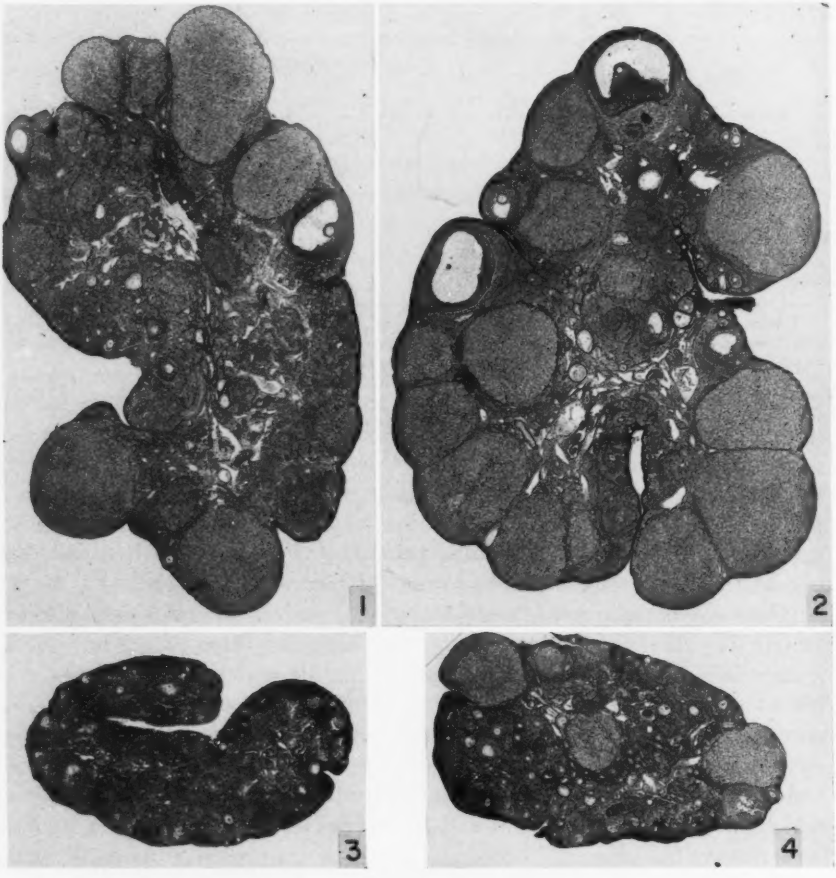
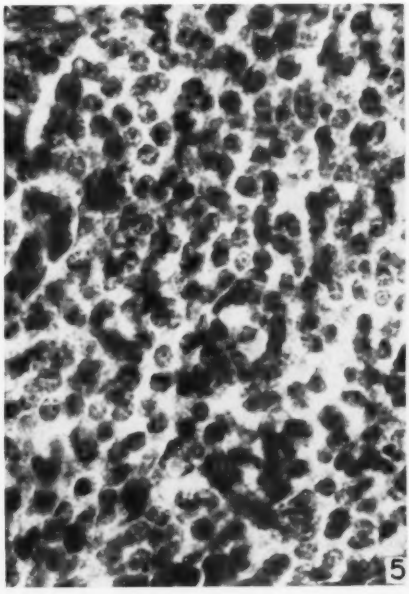


FIG. 1. Ovary of a normal cycling rat. Proestrus. 18×.

FIG. 2. Ovary of a cycling rat from group MEm-1, 98 days after re-transplantation of pituitary under the median eminence. A pregnancy had been terminated by caesarian section a month before autopsy; regular cycles had resumed thereafter. Proestrus. 18×.

FIG. 3. Typical atrophic ovary from group TL after re-transplantation of pituitary under the temporal lobe. Note absence of corpora lutea. 18×.

FIG. 4. Typical atrophic ovary from group Ky after pituitary transplantation to renal capsule. Small corpora lutea only, the result of transplanting during proestrus. 18×.



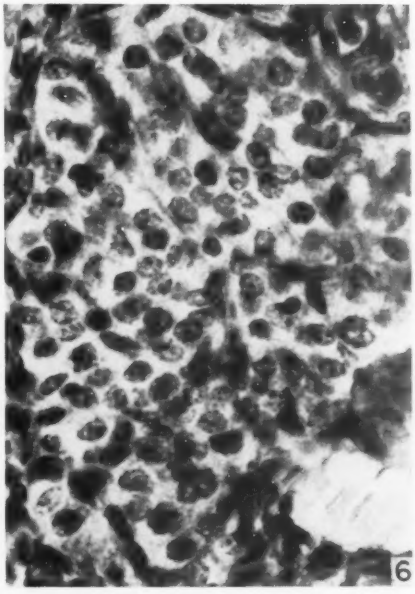


FIG. 5. Interstitial tissue from the atrophic ovary illustrated in Figure 4, showing marked LH deficiency. 715×.

FIG. 6. Interstitial tissue from the ovary illustrated in Figure 2, showing good repair by the re-transplanted pituitary. 715×.

degree of estrogenic stimulation of the reproductive tracts. No stimulation of follicle development was noted, however.

Uterus and vagina. These organs were completely restored in the cycling animals from groups MEm-1 and MEm-2 and showed the characteristic histologic picture of the stage of the cycle when the animal was killed (Figs. 7, 9). In groups TL and Ky both the uterus and the vagina were atrophic (Figs. 8, 10).

Adrenals. Table 2 shows that in animals from group MEm-1 the right adrenal weight was significantly greater than that of groups TL and Ky, although somewhat lower than normal. The left adrenal hypertrophy in the experimental animals was just about as great as in the normal, while in groups TL and Ky it was insignificant.

Histologic examination supplemented these findings. The adrenal cortex in the experimental group was thinner than that of normal controls but significantly thicker than in either of the other control groups (Fig. 11). In addition, it was seen that in the experimental group the cells of the fasciculata were well organized in columns, while this was not the case in the temporal lobe and kidney controls. The reticularis, which is well developed in normal adrenals, was not usually evident in glands from group

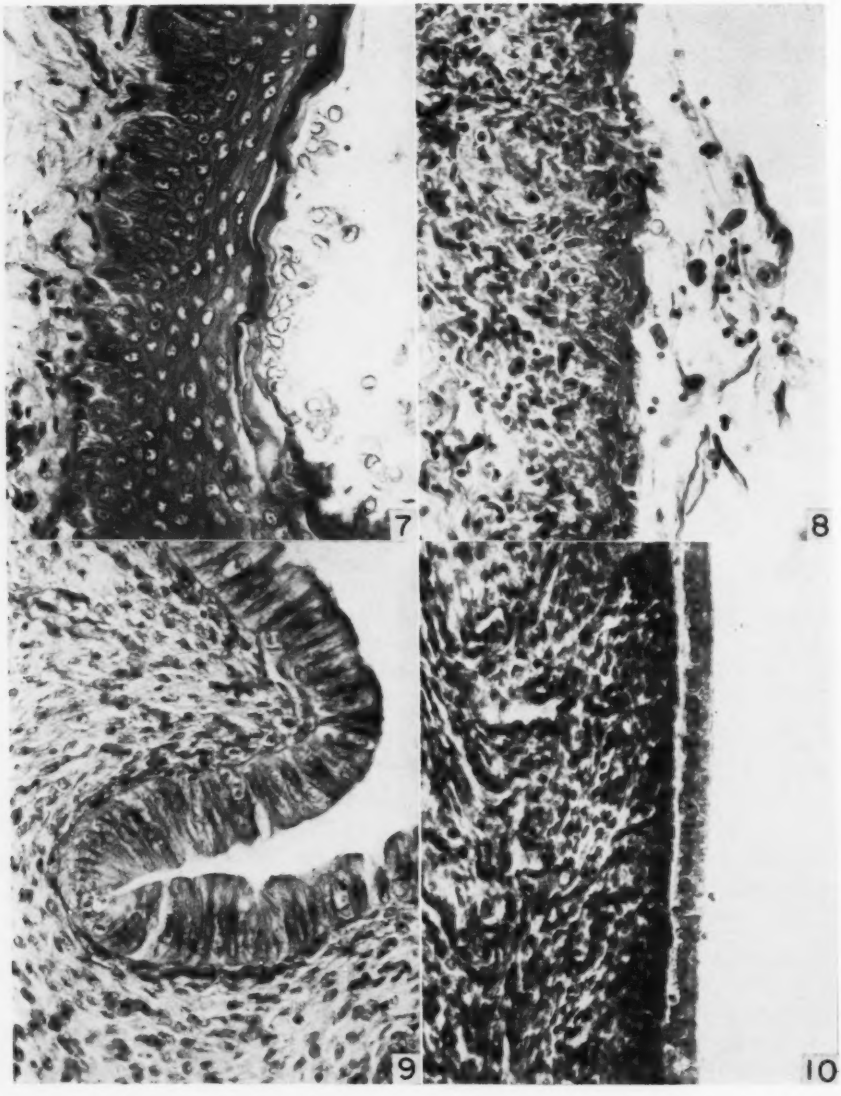


FIG. 7. Vaginal epithelium from a rat that cycled after re-transplantation of the pituitary under the median eminence. Proestrus. 300×.

FIG. 8. Atrophic vaginal mucosa from a temporal lobe control rat. 300×.

FIG. 9. Proestrous uterine mucosa from a rat bearing a pituitary re-transplanted under the median eminence. 300×.

FIG. 10. Atrophic uterine mucosa from a temporal lobe control rat. 300×.

TABLE 2. EFFECTS OF LOCATION OF THE PARS DISTALIS ON ADRENALS AND THYROIDS

Group	No. of rats	Rt. adr. wt. mg./100 g. (mean ±S.E.)	Lft. adr. hypertrophy 10 da. increase mg./100 g. (mean ±S.E.)	Thyroid wt. mg./100 g. (mean ±S.E.)	I^{131} uptake in 24 hrs. (mean ±S.E.)
TL	9	2.8±0.18	0.074±0.047	5.3±0.18	0.33±0.08
Ky	5	2.4±0.22*	0.41 ±0.15*	4.4±0.14*	1.20±0.09*
MEm-1	11	5.4±0.56*	1.62 ±0.27*	6.3±0.27	2.67±0.31*
N	10	9.1±0.42	2.36 ±0.30	6.5±0.20	6.99±0.83

* Differences are statistically significant ($P < 0.01$). Although in groups TL and Ky the means in the various categories also differ in similar degree by the "t" test, this may have no real meaning on account of the relative increase in operational error in the trimming of the small objects. Note that the advantage is with group TL in the first and third columns, but with group Ky in the second and fourth columns.

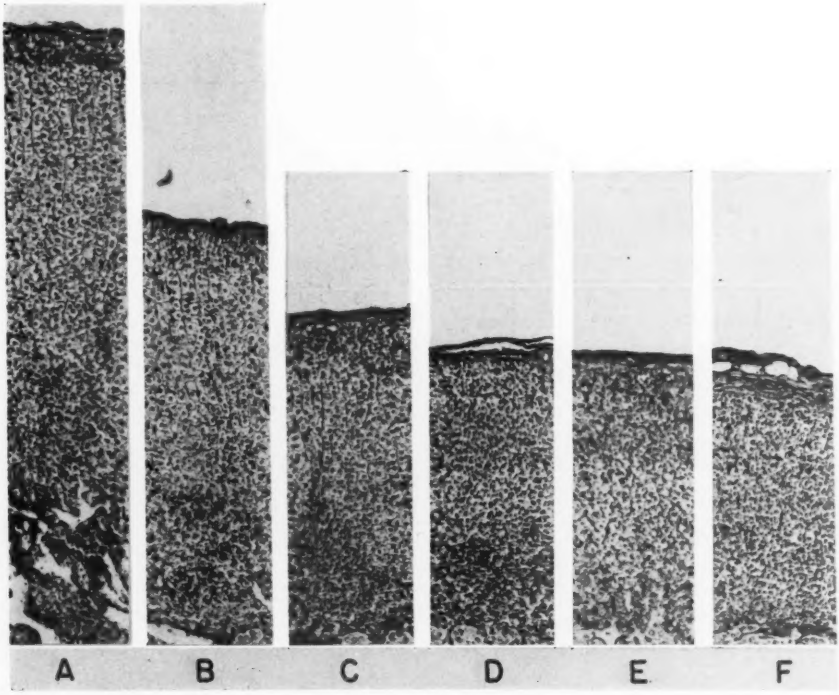


FIG. 11. Comparison of adrenal cortex histology from comparable regions of representative experimental and control rats. In each photo the medulla is just visible at the bottom. A—Normal, adult female, B, C—Maximum and minimum degrees of fasciculata restoration in rats from group MEm-1. D—Group Ky. E, F—Group TL. 100×.

MEm-1 (Fig. 11A, B). It was prominent, however, in adrenals from groups TL and Ky.

Thyroids. From Table 2 it can be seen that the thyroid weights of the experimental group MEm-1 and the control groups TL and N were very similar, while significantly lower in the control group Ky. The functional study with radioactive iodine brought out pronounced differences. The 24-hour uptake of I^{131} was lower than normal in group MEm-1, but significantly greater than that of either group TL or Ky.

Histologic examination showed that in the experimental group the glandular epithelium was low cuboidal, the cells being somewhat taller and less crowded than in the temporal lobe and kidney controls (Figs. 12, 13, 14, 15).

Body weight. The variation in body weight in groups MEm-1 and MEm-2 could not be well correlated with the apparent function of the grafts. Some rats gained a little while others lost as much as 30 grams. Those that became pregnant gained appropriately while carrying their litters, but promptly returned to their earlier weights afterward. In control group TL, nearly all animals lost weight following the second operation. Animals from control group Ky remained nearly at the weight they had at the time of the transplantation. Several lost a little and only a few were heavier.

Pituitary grafts. In all 24 animals from group MEm-2, even those that did not cycle, the pituitary grafts were very well placed under the median eminence and were adherent to it. This fact was easy to observe with the dissecting microscope at the time of autopsy and by histologic examination of *parasagittal* sections of the graft and overlying diencephalon (Fig. 16). It was also obvious on histologic examination that these grafts derived their main blood supply from the capillary plexus of the median eminence. Experience in the preliminary study (10) had shown that vascular injection of India ink was of no great value in demonstrating this point. Hence, no such injections were made in this definitive study. For photographic purposes, however, the ink-injected preparations have high value. One from a re-cycling animal in the preliminary series is shown in Figure 17, a parasagittal section through the hypothalamus, median eminence, stalk and graft.

All grafts under the median eminence appeared to be well vascularized. Their size seemed smaller than that of grafts in the renal capsule, although no exact measurements were taken. The grafts under the temporal lobe were usually as well vascularized, but appeared somewhat smaller than those under the median eminence. Grafts on the kidney were generally the largest. Like the others, they were always richly injected with blood.

Test for luteotropin secretion by grafts under the temporal lobe. It was noted that ovaries of all animals from group TL were devoid of large corpora lutea following the re-transplantation of the graft, regardless of whether the original transplantation had been performed during proestrus or at other

stages of the cycle. To determine whether these grafts were nevertheless secreting luteotropin, 8 animals were treated with FSH and LH to form new corpora lutea. Seven days following the end of the injection series (with one exception at a 4-day interval) uteri were traumatized with two loops of silk thread, and 4 days later the decidual response was evaluated on a scale of ++++.

At the time of traumatization, it was noted by examination of the ovaries that in 1 case no corpora lutea had been produced by the hormonal treat-

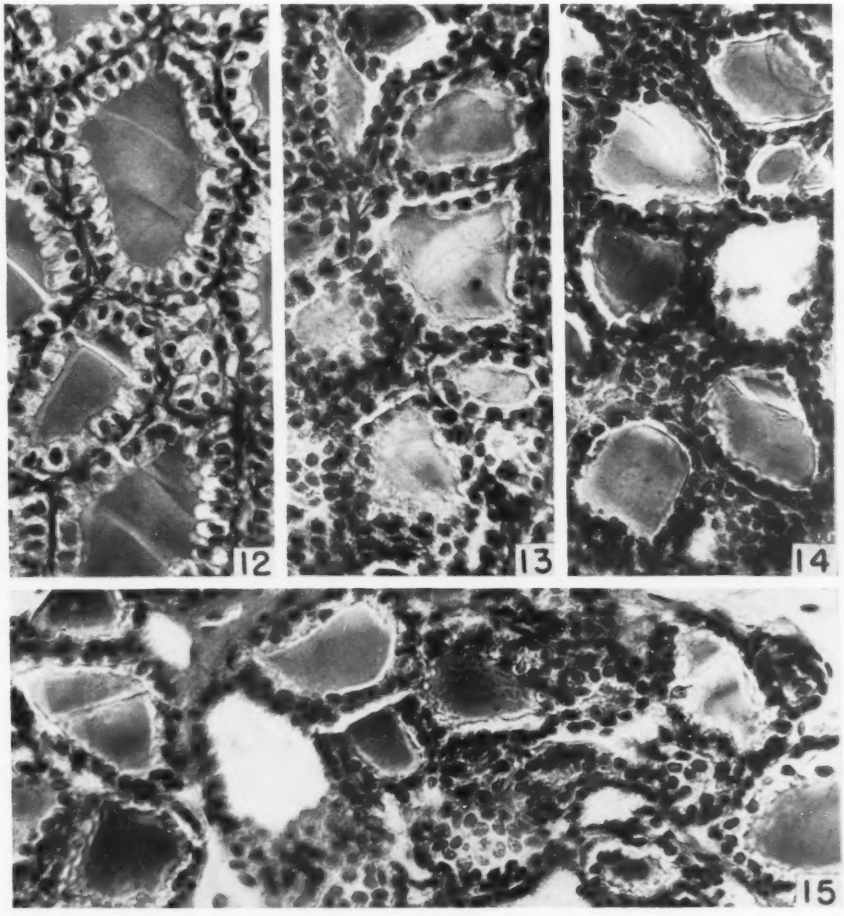


FIG. 12. Thyroid gland of normal control female rat. 400×.
FIG. 13. Thyroid gland from group MEm-1. 400×.
FIG. 14. Thyroid gland from group TL. 400×.
FIG. 15. Thyroid gland from group Ky. 400×.

ment. In the remaining 7 animals the corpora lutea were rather small, somewhat less than 1.5 mm. approximate diameter. No decidual reaction was obtained in 2 animals, including the one lacking corpora lutea, while in the remaining 6 animals reactions ranging from + to +++ were recorded. Thus, it was evident that their grafts were secreting luteotropin.

Proof of hypophysectomy. Serial sections of the hypophysial capsules from all animals in groups MEm-1 and MEm-2 were rigorously searched for remnants of the pars distalis. None was present in 9 animals from group MEm-1, including 4 fertile rats, 4 infertile cycling rats and the anestrous animal. In group MEm-2, 6 animals were free of remnants, including 3 persistent-estrous rats, 1 irregularly cycling rat and 2 of the anestrous ones. Only one rat in either of these groups, a fertile animal from group MEm-1, had a fragment that one might regard as large enough to contribute significantly to the end result. All other remnants were so small as to be of no consequence.

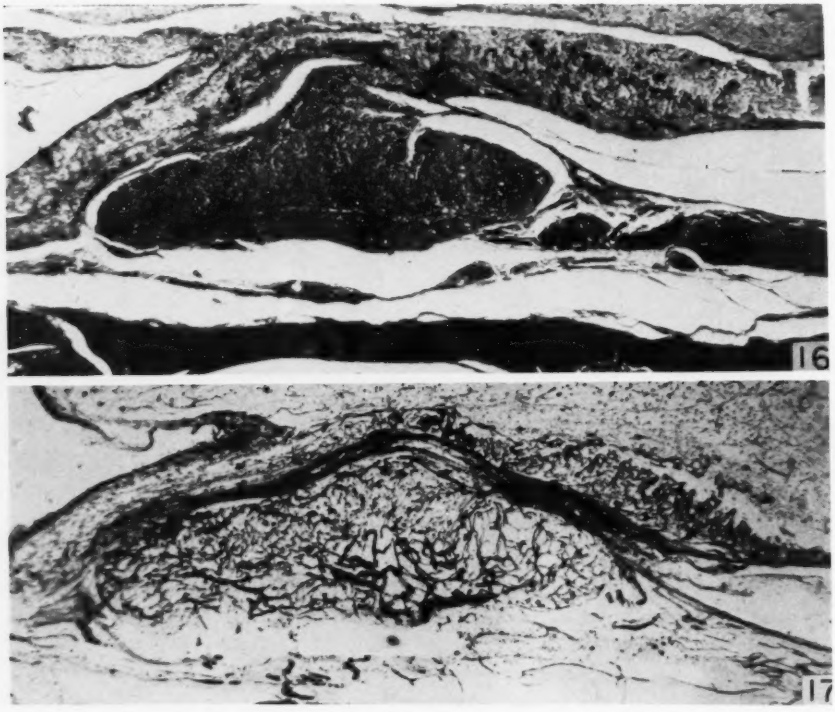


FIG. 16. Re-transplanted pituitary adherent to median eminence and stalk from an animal in which cycles had returned. 40×.

FIG. 17. Thick section (100μ) adjacent to the above, showing vascular pattern injected with India ink. Unstained. 40×.

The complete lack of FSH and LH activities in groups TL and Ky made it unnecessary to establish histologic proof of the absence of fragments in these cases.

DISCUSSION

Circumstances which distinguish the results of re-transplantation from those of direct transplantation of the fresh gland to the median eminence region immediately after hypophysectomy are (a) that the gland is twice insulted—severely so, in fact (7), and (b) that while in the kidney the gland undergoes an apparent functional de-differentiation with accompanying cytologic regression. It is all the more surprising and significant, therefore, to have encountered the high degree of functional restoration in the large proportion of animals in the median eminence groups.

This improved frequency of success in contrast to the small number in the preliminary experiments (9, 10) may be ascribed to greater uniformity achieved in placing the graft and, possibly, to the fact that special effort was made to strip off encapsulating connective tissue. In several failures of the preliminary series histologic examination of the graft and adjacent brain had disclosed a considerable sheet of connective tissue which might well have constituted a partial barrier.

The priming treatments proved to be of little value, either the small amounts of estrogen used in the preliminary series or the FSH-LH injections in group MEm-2. One may properly assume that in all animals in which cycling was restored this took place entirely spontaneously. The FSH-LH treatment appears to have been deleterious in one respect, seemingly having brought about persistent estrus in several cases. The reason is not apparent.

There was a detectable amount of functional recovery in even the four members of groups MEm-1 and MEm-2 that failed to cycle. Ovarian stimulation was evidenced by interstitial-cell repair in local areas, occasional theca luteinization and moderate estrogenic stimulation of the vagina and uteri. There was little indication of increased follicle development, however. The reason is not evident why these ovaries were not better stimulated, for the grafts were very well placed. There is a possibility of hypothalamic damage in the one failure of group MEm-1; the graft was imbedded in a cleft in the tuber.

Inspection of serial sections of the grafts disclosed no greater volume of parenchyma after re-transplantation under the median eminence than one usually finds in established grafts in the renal capsule. In fact, the trend was in the other direction. By contrast with the normal gland, the amount of parenchyma in even the best examples of the median eminence series was clearly rather small. This quantitative deficiency affords a ready explanation for the fact that ovarian weights, numbers of follicles and corpora lutea in each set, thyroid uptake of I^{131}, adrenal weight and

adrenal hypertrophy after hemiadrenalectomy were all less than in the normal rat. Only thyroid weights were equivalent. However, one must admit that the initial atrophy of the target tissues may have contributed to the end result by impairing their ability to respond.

Grafts under the temporal lobe were characteristically smaller than those on the kidney or under the median eminence. In this respect, they constitute less satisfactory controls than those left on the kidney. Nevertheless, several were shown to be competent sources of luteotropin, a fact which attests to their vitality, and there was no cytologic restoration to suggest changes in function from that prevailing in the kidney site.

It should now be fully evident that the extreme deficiencies observed when the pars distalis is removed to sites distant from the hypothalamus result from the loss of hypothalamic influences. The observations contribute additional strong evidence of humoral mediation of these stimuli, on the basis of the well documented opinion that the hypophysial portal circulation constitutes the principal link between median eminence and pars distalis (1, 15). The rapid onset of renewed gonadotropic function in a few animals of the median eminence series agrees with the known rapidity of regeneration of these portal venules (11).

Greater vigor of TSH and ACTH secretion in the median eminence groups, compared with controls bearing grafts on the kidney or under the temporal lobe, demonstrates that for even these functions the rat pars distalis depends importantly on hypothalamic stimuli. Our findings agree with others in this respect (16) and, on the other hand, with the numerous observations that a residual secretion of TSH and ACTH can proceed without close linkage of the pars distalis with the hypothalamus. Comparable residual secretion of FSH and LH, although not denied by this study, is clearly below levels that can be detected by the present methods.

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NOTES AND COMMENTS

ABSTRACT

The author has shown previously that the inhibitory effect of thyroxine on wound healing is mediated by a mechanism which is probably different from that of its action on the oxygen consumption. In this study the effect of *d,l*-thyroxine and a thyroxine dehalogenase-inhibitor n-butyl-4-hydroxy 3:5 diiodo-benzoate on the tensile strength of healing wounds was examined in guinea pigs. BHDB, which in itself has no effect on the wound healing, significantly enhanced the inhibitory effect of thyroxine on the tensile strength of wounds.

EFFECT OF THYROXINE AND A DEHALOGENASE-INHIBITOR ON THE TENSILE STRENGTH OF HEALING WOUNDS

Gross and Pitt-Rivers (1) as well as Roche *et al.* (2) showed that triiodothyronine (TIT) was a normally occurring substance and that it exerts a far more marked effect than thyroxine (THY)(= tetraiodothyronine) upon myxedema (3). It was known already that the normal animal organism contains dehalogenases which can convert THY to other iodinated compounds (4) which were later shown to be TIT. These dehalogenases, which are also found in athyrotic patients (5), appear to be extremely widespread, being present particularly in liver and kidney (6, 7, 8). The conversion of THY into TIT by deiodination appears to be necessary for THY to exert its action on oxygen uptake in man (9, 10, 11, 12).

The inhibitory effect of thyroxine upon the tensile strength of healing wounds which has been reported previously (13) did not, however, seem to be related to the same mechanism as that which causes an increased oxygen consumption (14). In an attempt to investigate whether the effect of thyroxine upon wound healing is due to a direct action or whether it requires deiodination to TIT, experiments were performed using a thyroxine-dehalogenase inhibitor, n-butyl 4-hydroxy 3:5 diiodo-benzoate (BHDB) which can completely inhibit the action of thyroxine upon oxygen uptake (15). In return, this substance potentiates the effect of triiodothyronine upon the oxygen uptake by inhibiting its further deiodination (16).

MATERIAL AND METHODS

The experiment comprised 38 male guinea pigs, weighing 281–440 gm. Under ether anesthesia two long incisions were made parallel with the midline of the dorsum, and always at the same distance from this line. The wounds were closed with continuous split nylon and left without dressing. Thereafter, the guinea pigs were divided into 4 groups. Six controls received intraperitoneal injections of physiological saline solution, 6 others received intramuscular injections of 30 mg. BHDB daily in an aqueous suspension, 13 received 40 μg. of *d,l*-thyroxine daily by the intraperitoneal route, and 13 received 40 μg. of *d,l*-thyroxine + 30 mg. of BHDB daily. Seven days after the incisions were made, the tensile strength of the wounds was measured at 3–5 different sites of the 2 wounds by the Sandblom, Petersen and Muren (17) tensiometer which permits measurements *in vivo* and *in situ*.

RESULTS

Table 1 gives the results of a total of 152 tensiometric determinations for 38 guinea pigs.

TABLE 1. TENSILE STRENGTH OF WOUNDS IN GUINEA PIGS TREATED WITH THYROXINE (THY) AND A THYROXINE-DEHALOGENASE INHIBITOR (BHDB)

	Controls (25)*	BHDB (23)	THY (52)	BHDB+THY (52)
Tensile strength in gm.	594 ± 24.1	622 ± 25.4	542 ± 18.7	473 ± 20.7

* Number of tensiometric determinations.

Statistical significance of the differences:
(1) Control/BHDB not significant.
(2) Control/THY not significant.
(3) Control/BHDB+THY $P<0.001$.
(4) BHDB/THY $0.02>P>0.01$.
(5) THY/BHDB+THY $0.02>P>0.01$.

The highest tensile strength was seen among the controls and among the guinea pigs that received BHDB. Among the thyroxine-treated guinea pigs these values were lower, but not significantly so except when compared with the animals which had received BHDB alone. The group which received thyroxine as well as BHDB showed values significantly lower than of the controls or of the animals which were treated with thyroxine.

DISCUSSION

These results agree with observations reported by others and indicate that thyroxine exerts an unmistakable effect also on metabolic mechanisms other than the oxygen consumption. Certain clinical findings in patients who appear to lack the thyroxine-dehalogenase show that thyroxine may increase the irritability and pulse rate, but that it cannot compensate the characteristic symptoms of myxedema (11, 12).

It has been demonstrated also that thyroxine—in addition to TIT and several other substances—has a marked uncoupling effect on the oxidative phosphorylation in hepatic mitochondria (18, 19). It is unlikely, however, that the action of thyroxine upon wound healing is due to a mechanism like the latter (20).

From the present results it may be concluded that thyroxine need not be deiodinated in order to exert its action upon the tensile strength of wounds. Upon administration of thyroxine together with a dehalogenase inhibitor there is an even more marked effect than caused by thyroxine alone, presumably because the thyroxine is not deiodinated and inactivated.

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THE ENDOCRINE SOCIETY

1959 Annual Meeting

The Forty-First Annual Meeting of The Endocrine Society will be held in the Haddon Hall Hotel, Atlantic City, New Jersey, Thursday, Friday, and Saturday, June 4, 5, 6, 1959.

The Chairman of Local Arrangements is Dr. Matthew Molitch.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily, and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 5th at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Chalfonte-Haddon Hall Hotel will hold 300 bedrooms for members until May 1, 1959, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations now directly with the hotel, advising them of time and date of arrival and departure. Make your reservations now and avoid disappointment.

Final program, membership card and advance registration forms will be sent on May 1st 1959 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Frank Engel, Duke Hospital, Durham, North Carolina, not later than February 1, 1959. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee. The Council requests that authors adhere strictly to the following rules when submitting abstracts, otherwise they cannot be considered:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.

Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be written after his name.

Line 3. Institution of origin and city in which institution is located.

4. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. There should be three copies.

5. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

1959 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and the Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1958.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by October 15, 1958.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.

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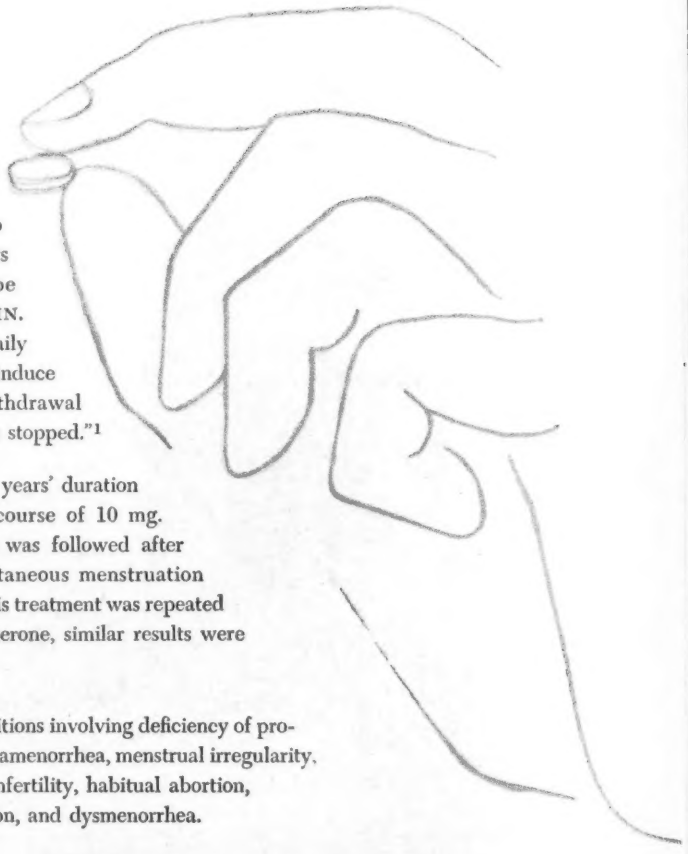

In gynecologic disorders amenable to progestational therapy, the clinical effects of injected progesterone can now be produced by small oral doses of NORLUTIN. In amenorrhea for example, 10-20 mg. daily for 5 days—after estrogen priming—will induce "...a prompt temperature rise and withdrawal bleeding 24-72 hours after medication is stopped."[1]

CASE SUMMARY[2] Amenorrhea of four years' duration in a 24-year-old married woman. A course of 10 mg. NORLUTIN, twice daily for five days, was followed after three days by menses. When no spontaneous menstruation occurred during the following 35 days, this treatment was repeated and again induced menses. Using ethisterone, similar results were unobtainable in this patient.

INDICATIONS FOR NORLUTIN: Conditions involving deficiency of progesterone such as primary and secondary amenorrhea, menstrual irregularity, functional uterine bleeding, endocrine infertility, habitual abortion, threatened abortion, premenstrual tension, and dysmenorrhea.

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(norethindrone, Parke-Davis)

oral progestational agent with unexcelled potency and unsurpassed efficacy

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REFERENCES:
(1) Greenblatt, R. B., & Jungck, E. C.: *J.A.M.A.* **166**:1461 (Mar. 22) 1958.
(2) Hertz, R.; Waite, J. H., & Thomas, L. B.: *Proc. Soc. Exper. Biol. & Med.* **91**:418, 1956.

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INSTRUCTIONS TO AUTHORS

Preference will be given to papers which are contributive to fundamental knowledge of the endocrines. The JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM, also published by The Endocrine Society, is held to be a more suitable outlet for papers on clinical endocrine medicine and case reports. Papers should be written with the greatest concision compatible with clarity and accuracy. Papers presenting negative or confirmatory results are considered only for the section on NOTES and COMMENTS. Submission of a paper to The Endocrine Society's journal is held to imply that it is not to be published elsewhere. Papers are published in the order of their acceptance with the exception that those given a low priority rating by the Editors may be delayed at the discretion of the Managing Editor.

NOTES and COMMENTS, a section appearing at the end of most issues is designed primarily for the prompt publication of material of unusual interest. Other papers suitable for this section include those in the nature of progress reports, those describing new methods, techniques or apparatus, and those describing results of a confirmatory or negative nature. Manuscripts must not exceed four double-spaced typewritten sheets. Tabular and illustrative material must be kept to a minimum.

NEW BOOKS will be listed as they are received and those which bear on topics related to endocrinology will be reviewed as promptly as possible and as space permits.

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1. Double-spaced or triple-spaced typewritten copy should be submitted in duplicate.
2. Tables, references, legends and footnotes should be on separate sheets.
3. In the text indicate citations to literature by number (in parentheses) and list references in numerical order at the end of the paper. Abbreviations of the names of journals should correspond to the system used by the Quarterly Cumulative Index Medicus. References should be typed as follows: Taurog, A., I. L. Chaikoff and D. D. Feller: *J. Biol. Chem.* 171: 1890. 1947. The references cited should have an important bearing on the work being reported.
4. The following type of abbreviation should be employed: gm., mg., μg., cc., m., cm., mm., μ, μμ, C., m.eq., R.U., U., I.U.
5. If the proprietary names of substances employed are to be given they should be appropriately identified as such.
6. Use "Fig." in parentheses and at the beginning of a legend; as a part of the text use "Figure," and "Table."
7. A substantive abstract in briefest possible form should accompany the article on a separate sheet. Do not send a summary.
8. Provide a running page title (not over 35 letters and spaces).

Photographs, preferably supplied unmounted, must be trimmed and arranged to conserve vertical space. Graphs or line drawings should be on white board or blueline paper. All lettering must be done in black ink and be suitably proportioned to print legibly. Photographs of graphs and drawings that are of inconvenient size for mailing will ordinarily be suitable. Colored photographs are published at the author's expense.

For each manuscript, authors will be allowed the equivalent of $25.00 for the reproduction of illustrative material and will be allowed one page of tabular material without charge. Important additional matter of this sort may be allowed at cost at the discretion of the Managing Editor. The cost of making excessive alterations in Galley proof will be charged to the author.

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Exactly how does new Halodrin* restore the "premenopausal prime" in postmenopausal women?

Webster defines "prime" as the period of greatest health, strength, and beauty. In a woman, these are the childbearing years between puberty and menopause—the years when her hormone production is highest.

The inevitable reduction in this hormone production as she enters the menopause often results in physical discomfort in the form of hot flushes, nervousness, insomnia, or a multiplicity of other symptoms with which you are familiar. Superimposed on this physical picture is the psychic trauma brought on by this unavoidable evidence of aging. The thing that brings her to a physician is simply that she "feels bad."

You can't make her 35 again—but the odds are good that you can make her feel like it! The secret is a combination of reassurance and hormones. The exact form and amount of the former defy objective analysis, but the latter can now be provided with scientific precision. Reduced to essentials, here is the explanation of exactly how hormones—in the form of Upjohn's new Halodrin—restore the "premenopausal prime."

The normal premenopausal woman excretes estrogens in the urine in the form of estradiol, estrone, and estriol, in an approximate 28-day average ratio of 39:15:46. Starting with this urinary excretion of estrogens, it is possible to calculate backwards and estimate the amount of estradiol that must have been secreted endogenously in order to produce these urinary levels. This is possible because the proportion of estrogens which appears in the urine following parenteral administration has been established in castrated women.

On this basis, the average endogenous output of estrogens is about 160 micrograms per day during a menstrual cycle, and 80 micrograms per day in postmenopausal women (see chart opposite). Therefore, the restoration of the "premenopausal prime" in the postmenopausal woman requires the replacement of approximately the equivalent of the 80 micrograms of estradiol per day that she no longer secretes endogenously.

Oral ethinyl estradiol is about 2 to 2½ times as potent as parenteral estradiol. Therefore, **the replacement of 80 micrograms of endogenous estradiol production per day is accomplished by the oral administration of 32 to 40 micrograms of ethinyl estradiol per day.**

Each Halodrin tablet contains 20 micrograms of ethinyl estradiol, which means that the recommended dosage of 2 tablets per day provides 40 micrograms of ethinyl estradiol. This offsets the loss of 80 micrograms of endogenous estradiol production in the menopausal woman; i.e., restores the "premenopausal prime."

Each Halodrin tablet also contains 1 mg. of Upjohn-developed Halotestin* (fluoxymesterone)—the most potent oral androgen known. The primary purpose is to "buffer" the ethinyl estradiol just enough to prevent breakthrough bleeding, which is obviously undesirable in the menopause. It also exerts other beneficial hormonal effects, one of which, in common with ethinyl estradiol, is a powerful anabolic action so desirable in patients of advanced years.

*TRADEMARK, REG. U. S. PAT. OFF.

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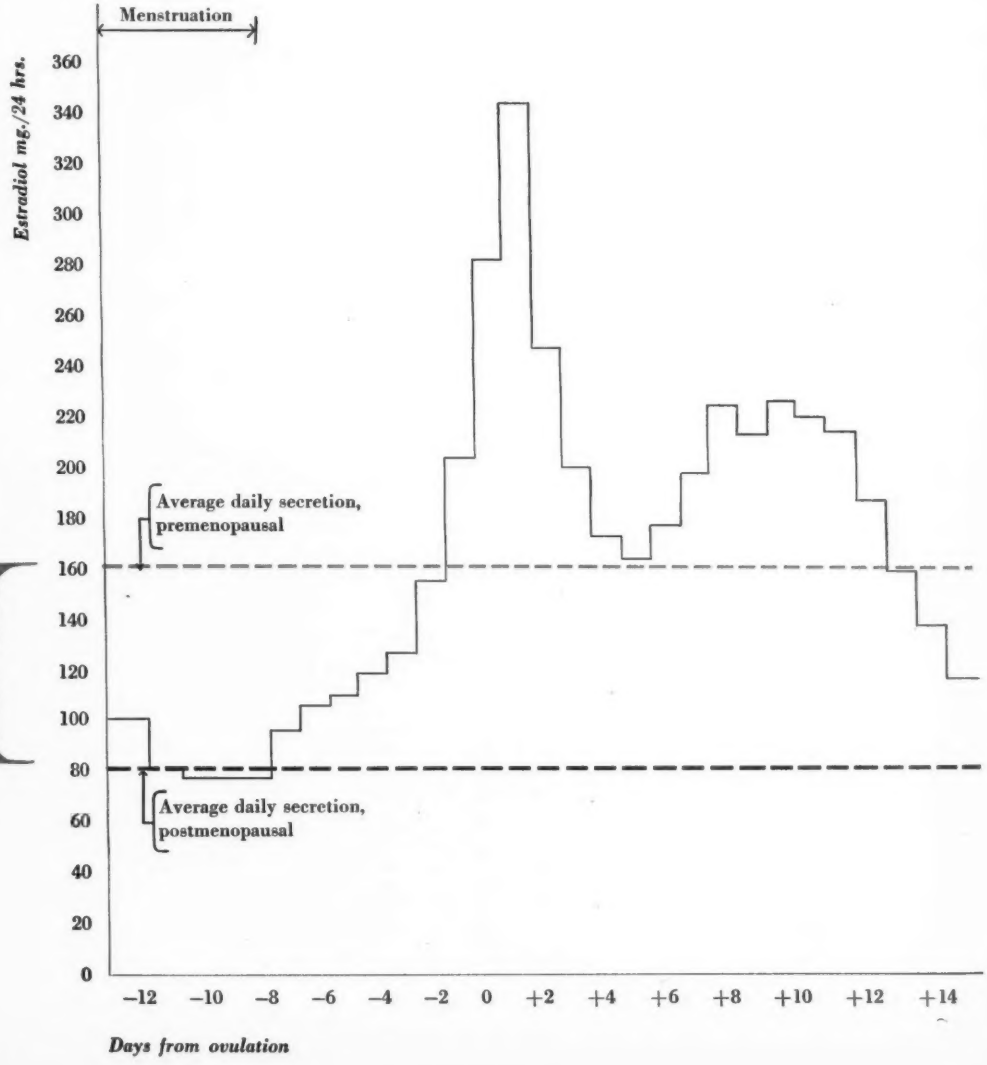
Upjohn

Endogenous estrogen secretion (mg./24 hours)
(calculated from average 24-hour urinary excretion of estradiol, estrone, and estriol)



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THE AMPHETAMINES
Their Actions and Uses

by
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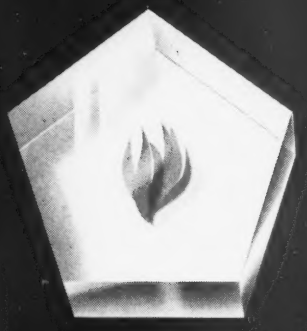

Decadron*

DEXAMETHASONE

to treat more patients more effectively

MERCK SHARP & DOHME
DIVISION OF MERCK & CO., INC.
PHILADELPHIA 1, PENNSYLVANIA

a new order of magnitude

in anti-inflammatory potency

DECADRON "possesses greater anti-inflammatory potency per milligram than any steroid yet produced,"[1] and is "the most potent steroid thus far synthesized."[2] Milligram for milligram, it is, on the average, 5 times more potent than 6-methylprednisolone or triamcinolone; 7 times more potent than prednisone; 28 times more potent than hydrocortisone; and 35 times more potent than cortisone.

in dosage reduction

Thanks to this unprecedented potency, DECADRON is "highly effective in suppressing the manifestations of rheumatoid arthritis when administered in remarkably small daily milligram doses."[3] In a number of cases, doses as low as 0.5-0.8 mg. proved sufficient for daily maintenance. The average maintenance dosage in rheumatoid arthritis is about 1.5 mg. daily.

in elimination and reduction of side effects

Virtual absence of diabetogenic activity, edema, sodium or water retention, hypertension, or psychic reactions has been noted with DECADRON.[1,2,3,4] Other "classical" reactions were less frequent and less severe. DECADRON showed no increase in ulcerogenic potential, and **digestive complaints were rare. Nor have there been any new or "peculiar" side effects,** such as muscle wasting, leg cramps, weakness, depression, anorexia, weight loss, headache, dizziness, tachycardia, or erythema. Thus DECADRON introduces **a new order of magnitude in safety,** unprecedented in corticosteroid therapy.

in therapeutic effectiveness

With DECADRON, investigators note "a decided intensification of the anti-inflammatory activity"[3] and antirheumatic potency.[4] Clinically, this was manifested by a **higher degree of improvement** in many patients previously treated with prednisteroids,[3] and by **achievement of satisfactory control in an impressive number of recalcitrant cases.**[3,4]

in therapeutic range

More patients can be treated **more effectively** with DECADRON. Its higher anti-inflammatory potency frequently brings relief to cases resistant to other steroids. Virtual freedom from diabetogenic effect in therapeutic dosage permits treatment of many diabetics without an increase in insulin requirements. Absence of hypertension and of sodium and fluid retention allows effective therapy of many patients with cardiovascular disorders. Reduction in the incidence and severity of many side effects extends the benefits of therapy to numerous patients who could not tolerate other steroids. And a healthy sense of well-being, reported by nearly all patients on DECADRON, assures greater patient cooperation.

References:
1. Boland, E. W.: California Med. 88:417 (June) 1958. 2. Bunim, J. J., et al.: Arthr. & Rheum. 1:313 (Aug.) 1958 3. Boland. E. W., and Headley, N. E.: Paper read before the Am. Rheum. Assoc., June 21, 1958, San Francisco, Calif. 4. Bunim, J. J., et al.: Paper read before the Am. Rheum. Assoc., June 21, 1958, San Francisco, Calif.

To treat more patients more effectively

in all allergic and inflammatory disorders

amenable to corticosteroid therapy

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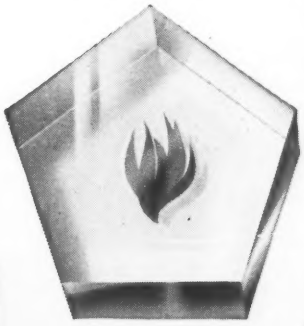
With proper adjustment of dosage, treatment may ordinarily be changed over to DECADRON from any other corticosteroid on the basis of the following milligram equivalence:

One 0.75 mg. tablet of Decadron* (dexamethasone) replaces:

one 4 mg. tablet of	one 5 mg. tablet of	one 20 mg. tablet of	one 25 mg. tablet of
methylprednisolone or triamcinolone	prednisolone or prednisone	hydrocortisone	cortisone

SUPPLIED:
As 0.75 mg. scored pentagon-shaped tablets; also as 0.5 mg. tablets, to provide maximal individualized flexibility of dosage adjustment.



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DEXAMETHASONE

Detailed literature on DECADRON is available to physicians on request.

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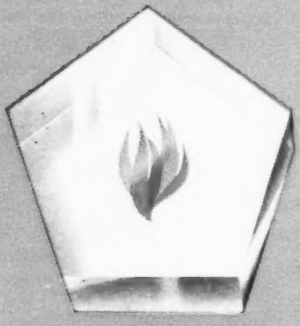

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Von

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H. J. HUSER, S. ROSIN, G. TRUOG,

MARIA SCHNEEBERGER, M. GEIGER

VIII + 234 p., 53 tables, sFr. 34.– / US $ 8.15

Separatum Vol. 7, No. 4 et Vol. 8, No. 1 «Acta Genetica et Statistica Medica»

This monograph represents a separate edition of three papers published in «Acta Genetica et Statistica Medica» in 1957 and 1958. It is published in German, but with an English Foreword, extensive English Summaries of its three parts and with English footnotes to all the 53 tables and figures containing the complete genetical, clinical and hematological data of the Study. In this way, the findings and the complete material are made accessible to the non-German speaking reader.

The subject of the Study is the investigation of the clinical, genetical and coagulation aspects of the Hemophilia B (PTC-Deficiency, «Christmas»-Disease) studied in the widespread and oldest described kindred of hemophiliacs, from the village of Tenna, Switzerland.

The authors present data on practically all descendants of the ancestral parent couple of this kindred, comprising 3072 persons, among whom 55 were hemophiliacs in 13 generations between 1600 and 1955. About 1500 descendants of the kindred were examined in the course of the five years of the investigation, among them 11 from the 13 hemophiliacs alive during the period. All genetical and clinical informations, the coagulation findings together with case histories and personal data, and the complete list of the sources of informations collected from the first part of the Study.

The second part of it gives statistical computation and genetical evaluation of the complete material for the fertility and mortality in the kindred of Tenna and for the fitness of bleeders and of the female carriers.

The third part of the Study is devoted mostly to the clinical manifestations of the disease and to the detailed coagulation studies of Tenna Hemophiliacs. The possibility of linkage of Hemophilia B in Tenna kindred with color blindness and with the ability to smell hydrocyanic acid is further evaluated. Finally, on the basis of the data on the Hemophiliacs from Tenna, an attempt is made to provide a general outlook on the genetical problems involved in the recent findings in coagulation physiology.

A mimeographed bilingual Register of Names, containing particulars of all 3074 persons covered by the Study, has been deposited in several European and American Genetic Institutes listed in the monograph.

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Present status of "A.P.L." therapy in cryptorchidism

TREATMENT IS CONSIDERED AT LOWER AGE LEVELS
HIGHER DOSAGE REGIMENS ARE NOW RECOMMENDED

Many clinicians advocate therapy with chorionic gonadotropin between the ages of 5 to 7; others suggest instituting treatment at ages ranging from 3 to 14.[1-8] Those practitioners who advocate therapy in early childhood or before puberty do so in the belief that a delay in treatment may result in irreversible aspermatogenesis. In most cases, 10 to 12 years would appear to be an appropriate age to start therapy. In addition, larger doses of chorionic gonadotropin than those formerly employed are now recommended on the basis that these will produce a greater incidence of satisfactory responses.[3]

Diagnostic use of "A.P.L."—A therapeutic trial with "A.P.L." will constitute a valuable aid to determine the need for surgery. Failure to respond may indicate an anatomic obstruction.

Definitive use of "A.P.L."—A large percentage of cryptorchid testes will descend following therapy with "A.P.L."

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Postoperative use of "A.P.L."—Follow-up therapy may be employed as an aid to preventing retraction of the testes.

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virtually painless on injection
practically free from local reactions

Suggested Dosage Regimens [depending on age]:

[1] 4,000 I.U., three times weekly, for two to three weeks, or
[2] 1,000 I.U., three times weekly, for six to eight weeks.

Caution: Evidence of sexual precociousness is an indication for the immediate withdrawal of treatment. After regression of undue development, reduced dosage regimen may be resumed.

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